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(54) Title: ANALOGS OF ACIDIC FIBROBLAST GROWTH FACTOR HAVING ENHANCED STABILITY AND BIOLOGICAL ACTIVITY

(57) Abstract

Analogs of proteins in the FGF family are provided in accordance with the present invention. These analogs are more stable than the corresponding naturally occurring proteins. Enhanced stability may be achieved by substituting at least one amino acid having a higher loop-forming potential for an amino acid residue having a lower loop-forming potential in an identified loop-forming region in the amino acid sequence of the protein. The analogs of the present invention are especially useful in therapeutic applications.

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ANALOGS OF ACIDIC FIBROBLAST GROWTH FACTOR HAVING ENHANCED STABILITY AND BIOLOGICAL ACTIVITY

Background

The complex process of healing which follows injury to tissue, such as by wounding or burning, is mediated by a number of protein factors sometimes referred to as soft tissue growth factors. These factors are required for the growth and differentiation of new cells to replace the destroyed tissue. Included within this group of soft tissue growth factors is a protein family of fibroblast growth factors (FGFs). The FGFs are mitogenic and chemotactic for a variety of cells of epithelial, mesenchymal, and neural origins. In addition, FGFs are angiogenic, that is they are able to stimulate the formation of blood vessels. Members of the FGF family include acidic FGF, basic FGF, KGF, Int-2, HST, FGF-5, and FGF-6.

Acidic FGF (aFGF) and basic FGF (bFGF) are considered to be two "original" members of the FGF family. Both aFGF and bFGF are believed to be derived from the same ancestral gene, with both molecules having approximately 55% sequence identity in addition to the same intron/exon structure. Acidic FGF and bFGF are also known to bind to the same receptor, although the existence of specific aFGF and bFGF receptors has not been ruled out. Several molecular weight forms of aFGF and bFGF are found in different tissues. However, Southern blotting experiments suggest that there is only one gene each for aFGF and bFGF, with differences between these molecules probably being due to post-translational processing. Both acidic and basic FGF are mitogens for a wide variety of cell types of mesodermal and neuroectodermal origin, and are able to induce

angiogenesis both *in vitro* and *in vivo* (see, e.g., Gospodarowicz *et al* (1979)), *Exp. Eye Res.*, 28:501-514. The range of biological activities of the two classes is nearly identical, although bFGF is about ten times more potent than aFGF in most bioassay systems.

KGF exhibits potent mitogenic activity for a variety of cells and it binds to cell surface receptors on Balb/MK keratinocytes to which aFGF and bFGF may also bind (Bottaro,*et al* (1990), *J. Biol. Chem.*, 265: 12767-12770. However, KGF is distinct from the known FGFs (*e.g.*, aFGF and bFGF) in that it is not mitogenic for fibroblasts or endothelial cells. Rubin *et al*, (1989), *Proc. Natl. Acad. Sci. USA*, 86: 802-806. KGF also has different receptors on NIH/3T3 fibroblasts from the receptors for aFGF and bFGF which fail to interact with KGF. Bottaro *et al*.

A shared distinguishing feature of aFGF and bFGF is the propensity of these factors to bind tightly to heparin. The affinity of aFGF for heparin appears to be weaker than for bFGF, with aFGF having an anionic isoelectric point (Thomas *et al* (1984), *Proc. Nat. Acad. Sci. USA*, 82:6409-6413. The unique heparin binding property of aFGF and bFGF has greatly facilitated purification of these factors.

The discovery that FGFs have strong affinity for immobilized heparin has also spurred investigation into the regulatory role of heparin-like molecules in the *in vivo* biology of FGFs. Although the full spectrum of functions for heparin has yet to be determined, it is known that heparin can regulate FGF function in several ways (Lobb (1988), *Eur. J. Clin. Invest.*, 18:321-326. For example, heparin-like molecules can play a direct role in FGF function, including the activation, or

potentiation, of aFGFs (Uhllrich *et al* (1986), *Biochem. Biophys. Res. Comm.*, 137:1205-1213.

There is, however, no direct correlation between the affinity of the FGF for immobilized heparin and its ability to be potentiated by soluble heparin. In this respect, the potentiating power of heparin appears to be selective for aFGF. For example, Uhllrich *et al.* (1986), *supra*, found the degree of potentiation of pure aFGF to be about ten times greater than that of pure bFGF, raising the potency of the aFGF to approximately the same level as that of bFGF. However, in the presence of fetal calf serum, the potentiating effect of heparin was found to decrease significantly (Uhllrich *et al.* (1986), *supra*).

The use of FGF proteins is believed to be effective in promoting the healing of tissue subjected to trauma. The unique angiogenic property of FGFs makes these factors especially valuable in the healing of deep wounds. The bFGF native proteins have been alleged to be useful in the treatment of myocardial infarction (U.S. Patents No. 4,296,100 and 4,378,347). In addition, human bFGF has been found to increase neuronal survival and neurite extension in fetal rat hippocampal neurons, suggesting that this factor may also be useful in the treatment of degenerative neurological disorders, such as Alzheimer's disease and Parkinson's disease (Wallicke *et al* (1986), *Proc. Natl. Acad. Sci. USA*, 83: 3012-3016).

A major stumbling block to the effective use of aFGF in therapeutic applications appears to be related to its significantly lower biological activity, as compared with bFGF. Although studies with heparin suggest that the observed difference in potency between

aFGF and bFGF can be substantially diminished by using heparin to boost the activity of aFGF to a level comparable to that of bFGF, the use of heparin in pharmaceutical preparations may not always be desirable. In this regard, it is important to note that heparin, a highly sulfated glycosaminoglycan of heterogeneous structure, is known to be an anticoagulant which functions by accelerating the rate at which antithrombin III inactivates the proteases of homeostasis (Jacques (1980), *Pharmacol Rev*, 31:99-166). It is not known whether it might be deleterious to use heparin in a pharmaceutical preparation for the treatment of deep wounds, where some degree of coagulation may be desired to achieve proper healing.

In addition, practical considerations can be expected to arise where heparin is incorporated into a pharmaceutical preparation for wound healing. Drug delivery concerns include the matter of controlling the composition of the pharmaceutical preparation (containing the combination of aFGF and heparin) upon entry into the patient's body. Moreover, the negative effect of fetal calf serum on the potentiating effect of heparin on aFGF (observed by Uhlrich *et al*) suggests that any advantage obtained by including heparin in the pharmaceutical preparation as an activating or potentiating factor for aFGF could be completely negated or lost once contact is made with the patient's own serum.

It is an object of the present invention to provide an analog of protein from the FGF family having enhanced stability as compared to the naturally occurring form of the protein It is a further object of the present invention to provide an analog of aFGF which exhibits enhanced stability and biological activity in

the absence of heparin. It is a further object of the present invention to provide an aFGF analog for therapeutic use.

Summary of the Invention

The present invention provides novel analogs of proteins in the FGF family. One such analog is an aFGF analog that is more stable and exhibits greater biological activity in the absence of heparin than naturally occurring aFGF. Another such analog is a KGF analog that has enhanced thermal stability as compared to naturally occurring KGF. Enhanced stability is achieved by substituting at least one amino acid having higher loop-forming potential for an amino acid residue of lower loop-forming potential in or about the loop-forming sequence Asn-His-Tyr-Asn-Thr-Tyr of the naturally occurring protein. In the case of aFGF, this loop-forming sequence occurs in the area of about amino acids 92 to 96. In the case of KGF, this loop-forming region occurs in the area of about amino acids 115-119. A preferred analog of the present invention incorporates the substitution of an amino acid having higher loop-forming potential for the histidine residue in the loop-forming sequence.

Brief Description of the Drawings

FIG. 1 shows the nucleic acid and amino acid sequences of recombinant bovine [Ala^{47},Gly^{93}] aFGF.

FIG. 2 show the amino acid sequence of recombinant human [Gly^{93}] aFGF.

FIG. 3 demonstrates the elution profiles for bovine [Ala^{47}] and [Ala^{47},Gly^{93}] aFGF analogs using hydrophobic interaction chromatography.

FIGS. 4A and 4B show the circular dichroic spectra for bovine [Ala^{47}] and [Ala^{47},Gly^{93}] aFGF analogs.

FIG. 5 shows the second derivative FTIR spectra of bovine [Ala^{47}] and [Ala^{47},Gly^{93}] aFGF analogs in the amide I' (C=O stretch in deuterated proteins) region.

FIG. 6 is a graph showing a plot of the log of the concentration of bovine [Ala^{47}] and [Ala^{47},Gly^{93}] aFGF analogs and human [Ser^{70},Ser^{88}] bFGF versus the percentage of maximal stimulation.

FIG. 7 is a graph showing the loss of activity over time of bovine [Ala^{47}] and [Ala^{47},Gly^{93}] aFGF analogs in the absence of heparin as compared with human [Ser^{70},Ser^{88}] bFGF.

FIG. 8 shows the structure of the bovine [Ala^{47},Gly^{93}] aFGF analog of the present invention, as determined by X-ray crystallography.

FIG. 9 shows the nucleic acid and amino acid sequences of naturally occurring KGF.

FIG. 10 shows the nucleic acid and amino acid sequences of recombinant [Gly^{116}] KGF.

Detailed Description of the Invention

Novel analogs of the FGF family are provided in accordance with the present invention. These analogs exhibit improved stability, as compared with the corresponding naturally occurring protein. In the case of the aFGF analog of the present invention, the analog exhibits enhanced stability and biological activity in the absence of heparin. In the case of the KGF analog of the present invention, the analog exhibits enhanced thermal stability. The analogs of the present invention have at least one different amino acid residue from the corresponding naturally occurring protein in or about the loop-forming sequence Asn-His-Tyr-Asn-Thr-Tyr found in the naturally occurring form. In the case of aFGF, the loop forming sequence occurs in the area of about amino acid residues 92 to 96 (based on the numbering of the known amino acid sequence for bovine aFGF, as shown in Fig. 1). In the case of KGF, the loop forming sequence occurs in the area of about amino acid residues 115-119, as shown in Figs. 9 and 10. The different amino acid(s) is selected for its higher loop-forming potential in order to stabilize this area of the analog. Amino acids having relatively high loop-forming potential include glycine, proline, tyrosine, aspartic acid, asparagine, and serine [Leszcynski *et al.* (1986), *Science*, 234:849-855 (1986) (relative values of loop-forming potential assigned on the basis of frequency of appearance in loop structures of naturally occurring molecules)]. Preferably, a different amino acid having higher loop-forming potential replaces the histidine residue in the loop-forming sequence. Still more preferably, the histidine in the loop-forming sequence is replaced with a glycine residue.

Other additions, substitutions, and/or deletions may be made to the analogs of the present invention. For example, the analog may also optionally include an amino acid substitution for non-conserved cysteine residues (*e.g.*, the cysteine residue at position 47 of the bovine aFGF molecule and the cysteine residue at position 16 of the human aFGF molecule). In addition, the analogs of the present invention which are expressed from *E. coli* host cells may include an initial methionine amino acid residue (i.e., at position -1, as shown in Fig. 1). Alternatively, one or more of the terminal amino acid residues may be deleted from the DNA sequence, as is known to those skilled in the art, while substantially retaining the enhanced biological activity of the corresponding naturally occurring protein.

DNA sequences coding for all or part of the analogs of the present invention are also provided. Such sequences preferably may include the incorporation of codons "preferred" for expression by selected *E. coli* host strains ("*E. coli* expression codons"), the provision of sites of cleavage by restriction endonuclease enzymes, and/or the provision of additional initial, terminal, or intermediate DNA sequences which facilitate construction of readily expressed vectors. These novel DNA sequences include sequences useful in securing the expression of the analogs of the present invention in both eucaryotic and procaryotic host cells, such as *E. coli*.

More specifically, the DNA sequences of the present invention may comprise the DNA sequence set forth in Fig. 1, wherein at least one codon encoding an amino acid residue in the area of about amino acids 92 to 96 is replaced by a codon encoding a different amino acid residue having a higher loop-forming potential

(hereinafter "aFGF analog sequence(s)" or "analog sequence(s)"), as well as a DNA sequence which hybridizes to one of the analog sequences or to fragments thereof, and, a DNA sequence which, but for the degeneracy of the genetic code, would hybridize to one of the analog sequences.

Correspondingly, the DNA sequences of the present invention may comprise the DNA sequence set forth in Fig. 10, wherein at least one codon encoding an amino acid residue in the area of about amino acids 115-119 is replaced by a codon encoding a different amino acid residue having a higher loop-forming potential (hereinafter "KGF analog sequence(s)" or "analog sequence(s)"), as well as a DNA sequence which hybridizes to one of the analog sequences or to fragments thereof, and, a DNA sequence which, but for the degeneracy of the genetic code, would hybridize to one of the analog sequences.

The analogs of the present invention can be encoded, expressed, and purified by any one of a number of recombinant technology methods known to those skilled in the art. The preferred production method will vary depending upon many factors and considerations, including the cost and availability of materials and other economic considerations. The optimum production procedure for a given situation will be apparent to those skilled in the art through minimal experimentation. The analogs of the present invention can be expressed at particularly high levels using *E. coli* host cells, with the resulting expression product being subsequently purified to near homogeneity using procedures known in the art. A typical purification procedure involves first solubilizing the inclusion bodies containing the analogs, followed by ion

exchange chromatography, then refolding of the protein, and finally, hydrophobic interaction chromatography.

The analogs of the present invention exhibit a surprising degree of enhanced stability. Unlike naturally occurring aFGF, the aFGF analogs of the present invention demonstrate enhanced stability and biological activity in the absence of heparin. While it is known that more stable bFGF analogs can be obtained through the substitution of serine or other neutral amino acids in place of certain cysteine residues (for example, as disclosed in published PCT Patent Application No. 88/04189), substitution for the non-conserved cysteine residue at position 47 of naturally occurring bovine aFGF alone is not believed to be significant in enhancing the biological activity and/or stability of an aFGF analog. This is demonstrated by the lower activity exhibited by a bovine [Ala^{47}] aFGF analog (cysteine substituted) compared with a bovine [Ala^{47},Gly^{93}] aFGF analog (having the desired amino acid substitution in the residue 92 to 96 region of the aFGF molecule), as set forth in the examples which follow. Specifically, the bovine [Ala^{47},Gly^{93}] analog, although still less potent compared with the bFGF, was found to be approximately ten times more potent than the bovine [Ala^{47}] aFGF analog. Upon the addition of 45 µg/ml heparin, bioactivity of all three forms of FGF was enhanced, with the bovine [Ala^{47},Gly^{93}] analog, the bovine [Ala^{47},Gly^{93}] analog, and human [Ser^{70},Ser^{88}] bFGF analog having substantially identical potency.

The reason for the enhanced mitogenic activity and stability of the bovine [Ala^{47},Gly^{93}] aFGF analog relative to bovine [Ala^{47}] aFGF in the absence of heparin was not immediately clear. The substitution of glycine for the histidine residue at position 93

appeared to make the aFGF molecule somewhat more hydrophobic, but it did not appear to drastically alter its tertiary structure, as determined by circular dichroism and FTIR spectroscopy. However, the relative differences in the activities observed in the *in vitro* bioassays for the bovine [Ala^{47},Gly^{93}] aFGF analog and for the bovine [Ala^{47}] aFGF analog (with substitution at only position 47) suggested that the glycine-substituted amino acid 93 position of the bovine [Ala^{47},Gly^{93}] aFGF analog might be within or near the region responsible for receptor binding. Although the receptor binding region in aFGF has not been determined, position 93 in aFGF corresponds to a region in bFGF which is reported to be within or near the receptor binding domain (Baird et *al* (1988), *Proc. Nat. Acad. Sci. USA*, 85:2324-2328.

In addition, the bovine [Ala^{47},Gly^{93}] aFGF analog of the present invention, unlike the bovine [Ala^{47}] analog, exhibited enhanced stability, maintaining its original mitogenic activity in the absence of heparin over the course of 250 hours, while the bovine [Ala^{47}] analog rapidly lost activity.

The bovine [Ala^{47},Gly^{93}] aFGF analog was crystallized, and the resulting crystals examined by X-ray crystallography. The X-ray crystallographic data obtained from examination of these crystals supports the suggestion from the hydrophobic interaction chromatography data that residue 93 is exposed to solvent, i.e., that the glycine for histidine substitution at position 93 makes the molecule less hydrophilic. Detailed examination of the bovine [Ala^{47},Gly^{93}] aFGF analog sequence around residue 93 revealed a clustering of approximately 8 amino acids with high loop-forming potentials in the region from about the glutamic acid residue at position 90 to about

the tyrosine residue at position 97. The relative loop-forming potentials of amino acids have been reported, with glycine being identified as the amino acid residue having the highest loop-forming potential among all amino acids (Leszcynski *et al* supra). Thus, the glycine for histidine substitution is believed to stabilize the presumed loop, due to the much higher loop-forming potential of the glycine residue compared with histidine.

The KGF analogs of the present invention also demonstrate that the corresponding region in KGF is a solvent-exposed loop that may be involved in receptor binding. Specifically, the [Gly^{116}]KGF analog of the present invention was found to exhibit an altered, decreased mitogenic activity compared to naturally occurring KGF, as set forth in the examples which follow. The [Gly^{116}] KGF analog was also found to have 5-7°C higher thermal stability relative to naturally occurring KGF.

Other analogs, in addition to the preferred [Gly^{93}] aFGF and [Gly^{116}] KGF analog specifically set forth herein, are contemplated by the present invention. These other analogs could easily be made by one skilled in the art by following the teachings provided herein. For example, there are no fewer than fifteen amino acids reported to have higher loop-forming potential than histidine (Leszcynski *et al*. (1986), *supra*). These amino acids are (in descending order of loop-forming potential) glycine, proline or tyrosine, aspartic acid or asparagine, serine, cysteine, glutamic acid, threonine, lysine, cystine, glutamine, arginine, phenylalanine, and tryptophan. Substitution of any of these residues for the histidine residue in the loop-forming sequence of the naturally occurring protein

could be expected to result in an analog of the present invention having an enhanced stability. Of course, it will be preferred to replace the histidine residue in the loop-forming sequence with amino acids having the highest possible loop-forming potential, without creating any potential negative effects, such as the formation of undesired disulfide bonds through the insertion of additional cysteine or cystine residues. Thus, other preferred amino acid substitutions at the histidine residue in the loop-forming sequence (*i.e.*, in addition to glycine) are seen to include proline, tyrosine, aspartic acid, asparagine, serine, glutamic acid, threonine, lysine, glutamine, arginine, phenylalanine, and tryptophan.

The present invention also contemplates the substitution of an amino acid having high loop-forming potential for other amino acid residues within the amino acid 92 to 96 region of naturally occurring aFGF (*i.e.*, amino acids 92 and 94-96) and the amino acid 115 to 119 region of naturally occurring aFGF (*i.e.*, amino acids 92 and 115-117-119). The aFGF analogs of the present invention include, for example, aFGF analogs having the threonine residue at position 96 of naturally occurring aFGF replaced with glycine, proline or tyrosine, aspartic acid or asparagine, serine, or glutamic acid, in order of preference, although minimal enhancement of stability and/or biological activity would be expected with the substitution of glutamic acid for threonine, due to the similarity of loop-forming potential of these two amino acids.

The amino acid residues at positions 92, 94, 95, and 97 (asparagine, tyrosine, asparagine, and tyrosine, respectively) of naturally occurring aFGF have sufficiently high loop-forming potential such that

minimal benefits are envisioned to arise from substitution for these particular residues.

The analogs of the present invention are seen to encompass analogs of both human and animal (e.g., bovine) origin, as well as all forms of a protein having the following loop-forming amino acid sequence:

```
                92  93  94  95  96   (aFGF)
            -Asn-His-Tyr-Asn-Thr-
               115 116 117 118 119   (KGF)
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For example, both the human and bovine forms of aFGF are known, and have been identified as having the identical amino acid sequence (shown above) at positions 92 to 96. Moreover, there is approximately 92% sequence identity between human and bovine aFGF, and a 97% "similarity" (i.e., 5% of the total 8% changes between the two aFGF forms are "conservative"). Both the human and bovine forms of naturally occurring aFGF exhibit substantially the same *in vitro* mitogenic activity.

Because of their enhanced stability and biological activity in the absence of heparin, the novel biologically active aFGF analogs of the present invention are particularly well suited for use in pharmaceutical formulations for the treatment by physicians and/or veterinarians of many types of wounds of mammalian species. The KGF analogs of the present invention, because of their enhanced thermal stability, may also be well suited for use in pharmaceutical preparations. The amount of biologically active analog used in such treatments will, of course, depend upon the severity of the wound being treated, the route of administration chosen, and the specific activity or

purity of the analog, and will be determined by the attending physician or veterinarian. The term "analog therapeutically effective" amount refers to the amount of analog determined to produce a therapeutic response in a mammal. Such therapeutically effective amounts are readily ascertained by one of ordinary skill in the art.

The analogs of the present invention may be administered by any route appropriate to the wound or condition being treated. Conditions which may be beneficially treated with therapeutic application(s) of the analog of the present invention include but are not limited to, the healing of surface wounds, bone healing, angiogenesis, nerve regeneration, and organ generation and/or regeneration.

The formulations of the present invention, both for veterinary and for human use, comprise a therapeutically effective amount of analog together with one or more pharmaceutically acceptable carriers therefore and, optionally, other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not be deleterious to the recipient thereof. The formulations may be conveniently presented in unit dosage form and may be prepared by any of the methods well-known in the art. All methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the analog with liquid carriers or finely divided solid carriers or both.

The following examples are provided to aid in the understanding of the present invention, the true

scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Example 1

Production of [Ala^{47},Gly^{93}] aFGF Analog

Synthesis

A [Ala^{47},Gly^{93}] aFGF analog and a [Gly^{116}] KGF analog were made using site-directed mutagenesis. It will be appreciated, however, that these and other analogs of the present invention can be made by other methods, including chemical synthesis. In the case of the [Ala^{47},Gly^{93}] aFGF analog, a bovine sequence was used. Specifically, the [Ala^{47},Gly^{93}] aFGF analog was prepared as follows:

A bovine aFGF analog according to the present invention was prepared and examined in the following examples. This analog, bovine [Ala^{47},Gly^{93}] aFGF, was constructed to contain both a desired amino acid substitution (glycine for histidine at position 93) in the residue 92 to 96 loop-forming sequence of the aFGF molecule and an additional amino acid substitution of alanine for the non-conserved cysteine residue at position 47, as shown in Fig. 1. A bovine [Ala^{47}] aFGF analog, having only the amino acid substitution of alanine for cysteine, was also prepared for use as a control for the desired bovine [Ala^{47},Gly^{93}] aFGF analog. Although these examples demonstrate a bovine aFGF analog of the present invention, the same results can be achieved for the highly homologous human aFGF analogs. For example, the amino acid sequence of the

corresponding human [Gly93] aFGF analog of the present invention is displayed in Fig. 2.

A synthetic gene coding for the [Ala47, Gly93] analog of bovine aFGF was assembled in two sections from a total of 28 component oligonucleotides. The amino acid sequence of Gimenez-Gallego *et al.* (1985), *Science*, 230:1385-1388 was used as the basis for this gene, with codon choices selected to optimize expression of the analog in *E. coli* (Gimenez-Gallego *et al.* (1985), *supra*). Section I was assembled from 16 oligonucleotides to yield a 287 nucleotide fragment which could be inserted into a plasmid vector at *Xba* I and *Xho* I restriction endonuclease sites. Section II was assembled from 12 oligonucleotides to give a 170 nucleotide fragment bounded by *Xho* I and *Bam* HI compatible ends. The two sections were inserted into the expression plasmid pCFM1156, which had been previously digested with *Xba* I and *Bam* HI in a 3-component ligation, yielding the complete aFGF gene under the control of the lambda pL promoter.

The plasmid pCFM1156 is prepared from the known plasmid pCFM836. The preparation of plasmid pCFM836 is described in U.S. Patent No. 4,710,473; the relevant portions of the specification, particularly examples 1 to 7, are hereby incorporated by reference. To prepare pCFM1156 from pCFM836, the two endogenous *Nde* I restriction sites are cut, the exposed ends are filled with T4 polymerase, and the filled ends are blunt-end ligated.

The resulting plasmid is then digested with *Cla* I and *Kpn* I and the excised DNA fragment is replaced with a DNA oligonucleotide of the following sequence:

5'-CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC-3'
3'-TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC-5'

E. coli cells transformed with this plasmid were grown in a 16-liter fermentation vessel (as described in Fox *et al* (1988), *J. Biol. Chem.*, 263:18452-18458.

The gene coding for the bovine [Gly^{93},Ala^{47}] aFGF was converted to the [Ala^{47}] form using oligo site-directed mutagenesis. The aFGF gene was first transferred into the phage vector M13mp18, and single-stranded DNA to serve as a template for the mutagenesis reaction was prepared. Approximately 0.5 µg of this DNA was mixed with 5 picomoles each of the mutagenic primer (5'-GAAGAAAACCATTACAACAC-3') and the M13 universal primer used for DNA sequencing, heated to 65°C for 3 minutes, and allowed to slow cool. The annealed template-primer was mixed with ATP, a dNTP mixture, DNA polymerase I large fragment, and T4 DNA ligase, then incubated at 15°C for 4 hours. Aliquots of this reaction mixture were added to competent *E. coli* JM101 cells and plated in 0.7% L-agar. The resulting plaques were replicated onto nitrocellulose filters and the filters were hybridized with ^{32}P-labeled mutagenic primer. DNA prepared from phage which hybridized was sequenced to verify successful completion of the desired mutagenesis event. The resultant gene was then transferred back to the pCFM1156 vector for expression of the recombinant protein.

Purification of aFGF analog

Both the bovine [Gly^{93},Ala^{47}] and [Ala^{47}] aFGF analogs were purified from the insoluble fraction obtained from centrifugation of mechanically lysed

E. coli cells expressing the recombinant protein. The pellet fraction was solubilized in 8 M urea, 0.1 M glycine, pH 2.5, and centrifuged to remove insoluble materials. The supernatant was loaded onto an S-Sepharose® (Pharmacia, Uppsala, Sweden) column equilibrated with 6 M urea, 10 mM glycine, pH 3.0, and washed with 6 M urea, 20 mM sodium citrate, pH 6.5. Proteins which bound to the column were eluted with a linear 0 to 0.5 M sodium chloride gradient in 20 mM sodium citrate, pH 6.5. The fractions containing the aFGF were pooled, diluted 20-fold with 20 mM sodium citrate, 0.1 M ammonium sulfate, and centrifuged to remove any precipitate. The supernatant was mixed with one volume of 20 mM sodium citrate, 2 M ammonium sulfate, and loaded onto a phenyl-Sepharose® column equilibrated with 20 mM sodium citrate, 1 M ammonium sulfate, pH 6.5. The bound proteins were eluted from the column with a linear descending gradient (1 M to 0 M) of ammonium sulfate. The aFGF-containing fractions were pooled and dialyzed against 20 mM sodium citrate, pH 6.5. This product was essentially homogeneous, as demonstrated by the fact that no other bands in Coomassie blue appeared in the SDS gel, as shown in Fig. 4.

Example 2

Gel Filtration Chromatography of aFGF Analog

Gel filtration was performed at room temperature using a Superose®-12 column on a Pharmacia FPLC system (Pharmacia, Uppsala, Sweden). The column was run at 0.5 ml/min in 20 mM sodium citrate, 0.2 M sodium chloride, pH 6.5.

Gel filtration chromatography showed that the purified bovine [Ala^{47}] and [Ala^{47},Gly^{93}] aFGF analogs eluted as single peaks at an elution position identical to that of ribonuclease A (M_r = 13,700). This indicated that both proteins are monomeric and have the same hydrodynamic radius, although there is a possibility that both forms of the protein interact with the column matrix and give a retarded elution from the column.

Example 3

Hydrophobic Interaction Chromatography of aFGF Analog

Hydrophobic interaction chromatography was performed at room temperature using a phenyl-Superose® column on a Pharmacia FPLC system. The sample, in 2 M ammonium sulfate, 20 mM sodium citrate, pH 6.5, was loaded onto the column which had been equilibrated with 2 M ammonium sulfate. After a 2 M ammonium sulfate wash, the remaining protein was eluted with an ammonium sulfate gradient descending from 2 M to 0 M, followed by a final wash with 20 mM sodium citrate, pH 6.5.

Because the elution position of a protein in hydrophobic interaction chromatography (HIC) is strongly dependent upon the exposure of hydrophobic regions in

the folded state, this technique provides a sensitive probe of the conformational homogeneity of similar proteins. Fig. 3 presents the elution profiles for the bovine [Ala^{47}] and [Ala^{47},Gly^{93}] aFGF analogs. The [Ala^{47}] aFGF showed a major peak eluting at 0.25 M ammonium sulfate, while the [Ala^{47},Gly^{93}] aFGF analog showed a single peak at 0.13 M ammonium sulfate, suggesting that both proteins exist primarily in a single distinct conformation. The elution at lower salt concentration by the [Ala^{47},Gly^{93}] aFGF indicates that it is slightly more hydrophobic than the [Ala^{47}] form. This observation is consistent with the replacement of the histidine residue at position 93 by glycine if the conformation of the protein is such that this residue is exposed to the solvent. Alternatively, the change in this residue could induce an overall change in the conformation of the molecule to produce a more hydrophobic structure.

Example 4

Spectroscopy of aFGF Analog

Circular Dichroism

Circular dichroic spectra were determined at room temperature on a Jasco Model J-500C spectropolarimeter (Jasco, Tokyo, Japan) equipped with an Oki If 800 Model 30 computer (Oki, Tokyo, Japan). Measurements were carried out at a band width of 1 nm using cuvettes of 1 and 0.02 cm for the near and far ultraviolet ranges, respectively. The data were expressed as the mean residue ellipticity, [θ], calculated using the mean residue weight of 113 for both forms of aFGF.

Circular dichroism (CD) spectra of the bovine [Ala^{47},Gly^{93}] and [Ala^{47}] aFGF analogs were nearly identical in both the far and near ultraviolet regions, as shown in Figs. 4A and 4B, respectively. The CD of the analogs were also very similar to the spectrum reported for human bFGF (Arakawa, *et al* (1989), *BBRC*, 161:335-341. The similarity of the spectra in the near ultraviolet region is consistent with similar tertiary structures for the FGFs.

Thermal Transition

The thermal transition of proteins was determined on a Response II spectrophotometer (Gilford, Medfield, Massachusetts) equipped with thermal programming and a thermal cuvette holder. Samples were heated at an increment of 0.1°C/min or 0.5°C/min and their absorbance monitored at 287 nm. Protein concentrations were determined spectrophotometrically using an extinction coefficient of 0.98 for bFGF and 1.04 for both bovine aFGF analogs at 280 nm for 0.1% protein.

Thermal denaturation of the aFGF analogs was examined in the presence and absence of heparin at both pH 6.5 and 7.0, 20 mM sodium citrate. In all cases, the proteins precipitated as the temperature was increased. The temperature at which the abrupt absorbance increase occurred was taken as the denaturation temperature. In the absence of heparin, this temperature was about 10°C higher for the bovine [Ala^{47},Gly^{93}] aFGF analog than for the bovine [Ala^{47}] aFGF analog. Addition of either 1.4-fold or 8-fold (w/w) excess of heparin increased the denaturation temperature for both forms by 14-20°C, depending upon the rate of temperature increase used. The difference between the denaturation temperature of

the two forms remained at about 10°C. There was no apparent effect of 1.4-fold or 8-fold (w/w) excess heparin on the CD spectra of either protein in the 240 to 340 nm range, although in the case of 8-fold excess heparin, the aFGF spectrum in the 240-260 nm region was masked by the absorbance of the heparin itself.

Fourier-transform Infrared (FTIR) Spectroscopy

Fourier-transform infrared (FTIR) spectra were determined to further examine the similarity in conformation of both aFGFs. For FTIR spectroscopy, the proteins were thoroughly dialyzed against water. Each protein was prepared as a 2% solution in a 20 mM imidizole buffer made in D_2O (Sigma Chemical Co., 99.9% isotopic purity). Solutions were placed in IR cells with CaF_2 windows and 100 μm spacers. For each spectrum, 1500 interferograms were collected and coded on a Nicolet 800 FTIR system equipped with a germanium-coated KBr beam splitter and a DTGS detector. The optical bench was continuously purged with dry nitrogen gas. Second derivative spectra were calculated (as described in Susi *et al* (1988), *Biochem. Biophys. Res. Comm.*, 115:391-397). A 9-point smoothing function was applied to the water vapor-subtracted spectra.

Fig. 5 shows the second derivative spectra of the [Ala^{47}] and [Ala^{47},Gly^{93}] bovine aFGF analogs in the amide I' (C=O stretch in deuterated proteins) region. For polypeptides and proteins, the frequencies of the component bands in this region are related to secondary structure content. Surewicz *et al*, (1988), *Biochem. Biophys. Acta*, 952:115-130. The spectra show strong bands at 1630 and 1685 cm^{-1} which are indicative of a significant amount of β-structures in the two proteins. A strong band near 1647 cm^{-1} is indicative of the

presence of irregular or disordered structures. The weaker peaks near 1666 and 1673 cm^{-1} arise from turn structures. A small peak is present near 1651 cm^{-1} in the spectra of both proteins. Amide I' components near this frequency are typically assigned to α-helices. However, it was recently shown that this band may arise from loop structures. Wilder *et al*, (1990), *Abstracts of the Fourth Symposium of the Protein Society, San Diego*. As shown in Fig. 5, the highly resolved FTIR spectra, unlike CD, clearly demonstrate the presence of β-structures and turns, and the spectra for bovine [Ala^{47}] aFGF analog and bovine [Ala^{47},Gly^{93}] aFGF analog are nearly superimposable, again suggesting that these two proteins have similar conformation.

The second derivative spectra showed apparently no difference in conformation between the two aFGF analogs. However, it was evident that deuteration of exchangeable protons occurred faster for the bovine [Ala^{47}] aFGF analog than for the [Ala^{47},Gly^{93}] analog during equilibration of lyophilized protein with D_2O solution. Since the two proteins have a similar conformation, the observed difference in H-D exchange rate cannot be explained from differences in the extent of exposure of exchangeable protons between them. It is more likely that the [Ala^{47}] aFGF analog has a more flexible structure, which renders amide protons more accessible to the solvent.

Example 5

Heparin Chromatography of [Ala^{47},Gly^{93}] aFGF Analog

Heparin-Sepharose® (Pharmacia) was packed into a 1 x 8 cm column and equilibrated with 10 mM Tris·HCl, pH 7.2. The column was loaded, washed with 10 mM

Tris·HCl, pH 7.2 and eluted with a linear gradient from 0 to 2.8 M sodium chloride in the same buffer at a flow rate of 0.5 ml/min using a Pharmacia FPLC system.

Acidic and basic FGF are distinguished by their avid binding to heparin and heparin-like molecules. Both the bovine [Ala^{47},Gly^{93}] and [Ala^{47}] aFGF analogs showed a single peak eluting at 1.54 M sodium chloride in 10 mM Tris·HCl, pH 7.2.

Example 6

Biological Activity of aFGF Analogs

In Vitro Bioassays

The mitogenic activity on NIH 3T3 cells of the aFGF analogs from the previous examples was determined as described below. In addition, a human [Ser^{70},Ser^{88}] bFGF analog, prepared as described in published PCT Patent Application No. 88/04189, was also examined in the bioactivity assays, alongside the aFGF analogs.

NIH 3T3 cells were obtained from ATCC. The cells were grown in DME supplemented with 10% calf serum, 10 units/ml penicillin, 2 mM glutamine and 10 units/ml streptomycin. Cells were passaged at a ratio of 1:40 two times per week. On day 1 of the assay, subconfluent cultures were trypsin dispersed and plated into 24-well plates at a concentration of 20,000 cells/ml, 1 ml per well in the above media. On day 5, the media was replaced with 1 ml/well DMEM without serum but containing penicillin, streptomycin, and glutamine at the above concentrations. On day 6, experimental samples were added to the media in volumes no greater

than 100 µl. Eighteen hours later, cells were pulsed for 1 hour with 1 ml of the above media containing 2-10 µCi of tritiated thymidine at 37°C. After the pulse, cells were washed once with media, then 250 mM sucrose, 10 mM sodium phosphate, 1 mM EDTA, pH 8 was added and the plates incubated at 37°C for 10 minutes to release the cells. Cells were harvested on a Skatron harvester (Skatron, Inc., Sterling, Virginia). Filters were dried, placed in scintillation fluid, and counted in a Beckman scintillation counter (Beckman Instruments, Inc., Fullerton, California).

The mitogenic activity of the bovine [Ala^{47},Gly^{93}] and [Ala^{47}] aFGF analogs on NIH 3T3 cells was examined as shown in Fig. 6. In the absence of heparin, the [Ala^{47}] aFGF analog produced a dose dependent stimulation of ^{3}H-thymidine uptake in the range of 1 to 100 ng/ml, with half-maximal stimulation of 25 ng/ml. Under the same assay conditions, the [Ala^{47},Gly^{93}] aFGF analog was able to produce the same mitogenic effect at a much lower protein concentration, the half-maximal dose being about 1 ng/ml. Recombinant bFGF was 4-5 times more potent than the [Ala^{47},Gly^{93}] aFGF, with a half-maximal dose of 220 pg/ml. When 4.5 µg/ml heparin was added to both analogs, their *in vitro* activity was increased with the [Ala^{47},Gly^{93}] aFGF analog remaining more potent. In the presence of 45 µg/ml heparin, the activities were enhanced such that the dose response of all three molecules were nearly identical, with a half-maximal dose of 90 pg/ml.

The stability of the aFGF analogs, as determined by retention of their respective mitogenic activity, was examined by incubation of a 0.1 mg/ml solution of each FGF analog in 20 mM sodium citrate, pH 7 at 37°C, both in the presence and absence of 1 mg/ml

heparin. In the absence of heparin, the bovine [Ala^{47}] aFGF analog rapidly lost activity, with a half-life of about 13 hours, as shown in Fig. 7. However, in the presence of heparin, bovine [Ala^{47}] aFGF lost no biological activity over the 250 hour course of the experiment. In contrast, neither bovine [Ala^{47},Gly^{93}] aFGF analog nor the human [Ser^{70},Ser^{88}] bFGF analog exhibited any loss of activity over the 250 hours, whether or not heparin was present.

Example 7

Crystallography of [Ala^{47},Gly^{93}] aFGF Analog

Crystals of bovine [Ala^{47},Gly^{93}] aFGF analog were grown by vapor diffusion against 0.2 M NH_4SO_4, 2 M NaCl, 0.099 M sodium citrate, and 0.02 M sodium potassium phosphate, pH 5.6. The protein droplet contained equal volumes of the reservoir solution and a 10 mg/ml protein solution. The crystals were trigonal (space group $P3_121$, a = 78.6 Å, c = 115.9 Å) and diffracted to 2.5 Å resolution. Intensity data were collected with a Siemens (Madison, Wisconsin) multiwire area detector mounted on an 18 kw rotating anode generator. The Siemens suite of processing programs was used for data reduction. Multiple isomorphous replacement (mir) phases were calculated to 3 Å resolution from two derivatives, with a figure of merit of 0.68. After solvent flattening, regions corresponding to two independent aFGF molecules in the asymmetric unit were identified. The general non-crystallographic symmetry relationships between these molecules were determined from rotation function, real-space translation function, and density correlation studies. A molecular envelope was defined around an averaged aFGF molecule with a modified B.C. Wang

algorithm. The phases were iteratively refined by molecular averaging and solvent flattening.

Initial maps revealed extended regions of β sheet structure that were truncated at the loops, due to a small molecular envelope, as shown in Fig. 8. The final map for model building was calculated with mir phases (from heavy atom parameters re-refined against averaged phases, as described in Rould *et al*, (1989), *Science*, 246:1135-1142) and iteratively averaged with a molecular envelope generated by placing 6 Å spheres about the atomic positions in the initial model. Averaging at 3 Å resolution converged to a final R-factor of 17.8% between the observed structure factors and structure factors calculated from the averaged and solvent flattening map. The graphic program TOM/FRODO, implemented for a Silicon Graphics 4D80 by C. Cambillau, was used to build residues 10 to 136 of the aFGF sequence into an averaged electron density map.

The crystallography results supported the hypothesis that the 90-97 region is involved in a loop structure. If this region is, in fact, involved in receptor binding (as suggested by Baird *et al*, (1988), *supra*) any amino acid substitution which stabilizes the loop may stabilize and/or enhance the biological activity of the molecule. This is presumably the mechanism for the observed activity enhancement attained with the bovine [Ala^{47},Gly^{93}] aFGF.

Example 8

Production of [Gly116] KGF Analog

Synthesis

In order to make the [Gly116] KGF analog, a coding sequence was first obtained for naturally occurring KGF and then altered at the codon for amino acid 116 in order to achieve a coding sequence for the analog.

The coding sequence for naturally occurring KGF was obtained using RNA isolated from human fibroblast cells (cell line AG1523) as a starting material from which to make cDNA for KGF using standard techniques known in the art. The KGF cDNA was then used as a template in polymerase chain reactions (PCR) to amplify the KGF gene. Due to the presence of an internal *Nde*I site in the KGF gene, the PCR DNA was made as two fragments that were then linked together at a unique *Bsm*I site. Oligonucleotides 238-21 and 238-24 (shown below) were used to make a DNA product that was subsequently cut with *Bsm*I and *Bam*HI and then isolated to yield a 188 base pair fragment of KGF. Oligonucleotides 238-22 and 238-24 were used to make a DNA product that was subsequently cut with *Nde*I and *Bsm*I to yield a 311 base pair fragment of KGF. The two DNA fragments, when ligated together create the gene for naturally occurring KGF, shown in Fig. 9.

In order to obtain a coding sequence for the desired [Gly116] KGF analog, it was necessary to substitute a glycine codon for the His116 codon in the KGF gene. This was achieved using PCR overlapping oligonucleotide mutagenesis with oligonucleotides 315-17

and 315-18, encoding the KGF DNA sequence corresponding to the His^{116} region with the appropriate base pair changes to encode the [Gly^{116}] KGF analog. The KGF DNA template for the PCR was the same as shown in Fig. 9, except that the DNA sequence between the *KpnI* and *EcoRI* sites was replaced by chemically synthesized DNA as shown in Fig. 10. Any convenient oligonucleotides corresponding to KGF DNA regions 5'- and 3'- of the site-directed mutational change, such as oligonucleotides 238-22 and 238-24 could be used to provide the outside primers for the overlapping mutagenesis PCR. An *EcoRI* to *BamHI* DNA fragment containing the [Gly^{116}] KGF analog coding sequence was then ligated into the previously described expression plasmid pCFM1156 already containing the KGF gene, so as to replace the corresponding region of the KGF gene with a region of the coding sequence containing the necessary changes to encode the [Gly^{116}] KGF analog (Fig. 10). The ligation DNA was then transformed into an FM5 (ATCC# 53911) host and colonies were isolated that contained the pCFM1156 [Gly^{116}] KGF analog plasmid. The FM5/pCFM1156 KGF [Gly^{116}] KGF analog strain was then fermented and cell paste harvested using standard fermentation techniques.

oligo 238-21	5'-ACAACGCGTGCAATGACATGACTCCA-3'
oligo 238-22	5'-ACACATATGTGCAATGACATGACTCCA-3'
oligo 238-24	5'-ACAGGATCCTATTAAGTTATTGCCATAGGAA-3'
oligo 315-17	5'-GGAAAACGGTTACAACACATATGCA-3'
oligo 315-18	5'-GTGTTGTAACCGTTTTCCAGAATTAG-3'

Purification of [Gly^{116}] KGF Analog

The cells containing the [Gly^{116}] KGF analog from the fermentation described above were first broken by suspending 665 g of the *E. coli* cell paste in *ca.* 4 L

20 mM sodium phosphate, pH 6.8, 0.2 M NaCl and then passing the suspension 3 times through a Gaulin Homogenizer at 9,000 psi. The suspension was then centrifuged in a Beckman JA-10 rotor (Beckman Instruments, Fullerton, California) at 10,000 rpm, for 30 minutes at 4°C.

Ion exchange chromatography was performed by applying supernatant from the centrifuged suspension to an S-Sepharose® Fast Flow (Pharmacia, Uppsala, Sweden) column (5 x 23 cm., 450 ml total volume) equilibrated with 20 mM sodium phosphate, pH 7.5, 0.2 M NaCl, at a flow rate of 25 ml/min. The column was then washed with 2 L of 20 mM sodium phosphate, pH 7.5, 0.4 M NaCl, and the [Gly116] KGF analog eluted with a linear gradient of 0.4 M to 0.6 M NaCl in 20 mM sodium phosphate, pH 7.5. Total gradient volume was 7 L (about 16 times column volume). Fractions containing KGF were pooled and concentrated about 22-fold over a YM®-10 membrane (Amicon) in a 400 ml stirred cell.

Size exclusion chromatography (SEC) was performed by applying half of the volume of the concentrated KGF (total volume of 80 ml) obtained from ion exchange chromatography to a Sephadex® G-75 (Pharmacia) column (4.4 x 85 cm, total volume of 1300 ml) equilibrated with 20 mM sodium phosphate, pH 6.8, 0.5 M NaCl and developing the column with this buffer. The process was then repeated with the second half of the concentrated KGF preparation.

A second ion exchange chromatography procedure was then performed by first pooling SEC fractions corresponding to the monomeric form of [Gly116] KGF analog, then diluting the pooled fractions with five volumes of 20 mM sodium phosphate, pH 6.8, 0.2 M NaCl

and applying the diluted fractions to an S-Sepharose® Fast Flow (Pharmacia) column (5 x 23 cm., 450 ml total volume) equilibrated with 20 mM sodium phosphate, pH 6.8, 0.4 M NaCl. This column was then washed with about 1.5 L of 20 mM sodium phosphate, pH 6.8, 0.4 M NaCl. The purified [Gly^{116}] KGF analog was eluted with a linear gradient of 0.4 M to 0.6 M NaCl in 20 mM sodium phosphate, pH 6.8. Total gradient volume was 10 L (about 22 times column volume). Samples containing the [Gly^{116}] KGF analog, as ascertained by SDS-PAGE, were pooled and the KGF content was determined by UV absorption.

Example 9

Spectroscopy of KGF Analog

Fourier-transform Infrared (FTIR) Spectroscopy

Samples were prepared for infrared spectroscopy by diafiltering protein solutions in 20 mM sodium phosphate, 0.5 M NaCl, pH = 6.8 into a 20 mM sodium phosphate, 0.15 M NaCl, pD = 6.8 buffer prepared in D_2O (Sigma, 99.9%+ isotopic purity). The pD values were determined by adding 0.45 to the pH reading from a glass electrode pH meter, according to Covington *et al.* (1968), *Anal. Chem.*, 40:700-706. The final protein concentration was 30 mg/ml. Protein solutions were placed in IR cells with CaF_2 windows and 100 µM Teflon spacers.

For structural characterizations, 256 double-sided interferograms were co-added and Fourier-transformed after application of a Happ-genzel apodization function using a Nicolet 800 FTIR system. The resolution was set at 2 cm^{-1}. Derivative spectra

and Fourier self-deconvolutions were performed according to Susi and Byler (1983), *Biochme. Biophys Res. Comm.*, 115:391-397, using the Nicolet software. Curve fitting was performed using program Peakfit™ (Jandel Scientific Co.). The infrared spectra of KGF showed strong similarity to those of bFGF and aFGF, indicating similar structures for these three proteins.

Thermal stability studies of the naturally occurring KGF and the [Gly116] KGF analog were performed by placing the IR cells in an electric heating jacket controlled by an automatic temperature controller (Specac Inc., Fairfield, Connecticut). The temperature was increased at a rate of 0.5°C/minute. IR spectra were collected at 8 cm^{-1} resolution.

At temperatures below 50°C, the spectra appeared to change little from the spectrum at ambient temperature. At temperatures greater than 50°C, the spectra indicated that the naturally occurring KGF undergoes a thermotropic transition at this point. Peaks near 1616 and 1685 cm^{-1} were evident in the spectra and, by 65°C, these peaks dominated the spectra with a corresponding loss of intensity at 1643 cm^{-1}. This spectral transition represents the cooperative unfolding of naturally occurring KGF. The observed thermal transition was not reversible, most likely due to aggregation of the unfolded protein. The melting temperature, T_m, for naturally occurring KGF was estimated to be 60°C, while the T_m for the [Gly116] KGF analog was estimated to be 65°C, 5°C higher than that of naturally occurring KGF, indicating that the [Gly116] KGF analog has a higher relative thermal stability than naturally occurring KGF.

Ultraviolet Spectroscopy

The thermal denaturation of both the [Gly^{116}] KGF analog and naturally occurring KGF was studied using a Response II UV spectrophotometer (Gilford, Medfield, Massachusetts) with a Peltier temperature controller and thermal programmer. KGF solutions in 20 mM sodium phosphate, pH 6.8, were mixed with 8 M guanidine HCl or 20 mM sodium phosphate for a final protein concentration of about 0.5 mg/ml and a final guanidine HCl concentration of 0 to 2 M. The thermal scan rate was set at 0.5°C/minute, and the wavelength monitored was 286 nm. Addition of a small amount of guanidine HCl eliminated precipitation of the proteins during thermal denaturation, but did not make the denaturation reversible. Therefore, samples to be compared were run simultaneously.

Thermal denaturation experiments using UV spectroscopy were unsuccessful in the presence of 0 and 1 M guanidine HCl, due to the development of turbidity upon heating. Nevertheless, turbidity developed at a lower temperature for the naturally occurring KGF than for the [Gly^{116}] KGF analog, suggesting that the former protein denatures and, consequently, aggregates at a lower temperature. In 1.5 M guanidine HCl, the absorbance at 287 nm decreased as the protein denatured and then increased due to aggregation. The temperatures at which the absorbance decrease and increase occurred were respectively 25 and 44°C for the [Gly^{116}] KGF analog. Addition of 2 M guanidine HCl resulted in partial denaturation for both the naturally occurring KGF and the [Gly^{116}] KGF analog at 25°C. Increasing the temperature resulted in complete denaturation for the proteins, denaturation ending around 37°C for naturally occurring KGF and 46°C for the [Gly^{116}] KGF analog.

These results indicate that the [Gly116] KGF analog is more thermally stable by several degrees in melting temperature, and are in agreement with the infrared analysis indicating an approximately 5°C increase in T_m of the [Gly116] KGF analog relative to naturally occurring KGF.

Example 10

Mitogenic Activity of [Gly116] KGF analog

Mitogenic activity of the [Gly116] KGF analog was analyzed using the mitogenesis assay of Rubin *et al.* (1989), *Proc. Natl. Acad. Sci. USA*, 86:802-806. The [Gly116] KGF analog demonstrated less 3H-thymidine incorporation than was observed for naturally occurring KGF, indicating a lower specific activity for the [Gly116] KGF analog.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Amgen Inc.

(ii) TITLE OF INVENTION: Analogs of Acidic Fibroblast Growth Factor Having Enhanced Stability and Biological Activity

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Amgen Inc
(B) STREET: 1840 DeHavilland Drive
(C) CITY: Thousand Oaks
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 91320-1789

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 463 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGAAAAA ACCAAGGAGG TAATAAATAA TGTTCAACCT GCCGCTGGGT AACTACAAAA 60

AACCTAAGCT TCTGTACTGC TCTAACGGCG GTTACTTCCT GCGCATTCTC CCGGATGGCA 120

CTGTAGACGG TACCAAAGAT CGTTCCGACC AGCACATTCA GCTCCAGCTC GCTGCAGAAT 180

CTATCGGTGA AGTTTACATC AAATCCACCG AAACTGGTCA GTTCCTGGCT ATGGATACTG 240

ATGGTCTCCT CTACGGTTCT CAGACTCCGA ACGAAGAGTG CCTGTTCCTC GAGCGTCTGG 300

AAGAAAACGG TTACAACACC TACATCTCCA AAAAACACGC TGAAAAACAC TGGTTCGTTG 360

GTCTGAAAAA AAACGGTCGT TCTAAACTGG GTCCGCGCAC TCACTTCGGT CAGAAAGCTA 420

TCCTGTTCCT CCCTCTGCCG GTTTCTTCCG ATTAATAGGA TCC 463

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 141 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Phe Asn Leu Pro Leu Gly Asn Tyr Lys Lys Pro Lys Leu Leu Tyr
1 5 10 15

Cys Ser Asn Gly Gly Tyr Phe Leu Arg Ile Leu Pro Asp Gly Thr Val
20 25 30

Asp Gly Thr Lys Asp Arg Ser Asp Gln His Ile Gln Leu Gln Leu Ala
35 40 45

Ala Glu Ser Ile Gly Glu Val Tyr Ile Lys Ser Thr Glu Thr Gly Gln
50 55 60

Phe Leu Ala Met Asp Thr Asp Gly Leu Leu Tyr Gly Ser Gln Thr Pro
65 70 75 80

Asn Glu Glu Cys Leu Phe Leu Glu Arg Leu Glu Glu Asn Gly Tyr Asn
85 90 95

Thr Tyr Ile Ser Lys Lys His Ala Glu Lys His Trp Phe Val Gly Leu
100 105 110

Lys Lys Asn Gly Arg Ser Lys Leu Gly Pro Arg Thr His Phe Gly Gln
115 120 125

Lys Ala Ile Leu Phe Leu Pro Leu Pro Val Ser Ser Asp
130 135 140

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 155 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ala Glu Gly Glu Ile Thr Thr Phe Thr Ala Leu Thr Glu Lys Phe
1 5 10 15

Asn Leu Pro Pro Gly Asn Tyr Lys Lys Pro Lys Leu Leu Tyr Ala Ser
20 25 30

Asn Gly Gly His Phe Leu Arg Ile Leu Pro Asp Gly Thr Val Asp Gly
35 40 45

Thr Arg Asp Arg Ser Asp Gln His Ile Gln Leu Gln Leu Ser Ala Glu
50 55 60

Ser Val Gly Glu Val Tyr Ile Lys Ser Thr Glu Thr Gly Gln Tyr Leu
65 70 75 80

Ala Met Asp Thr Asp Gly Leu Leu Tyr Gly Ser Gln Thr Pro Asn Glu
85 90 95

Glu Cys Leu Phe Leu Glu Arg Leu Glu Glu Asn Gly Tyr Asn Thr Tyr
100 105 110

Ile Ser Lys Lys His Ala Glu Lys Asn Trp Phe Val Gly Leu Lys Lys
115 120 125

Asn Gly Ser Cys Lys Arg Gly Pro Arg Thr His Tyr Gly Gln Lys Ala
130 135 140

Ile Leu Phe Leu Pro Leu Pro Val Ser Ser Asp
145 150 155

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 502 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATGTGCAAT GACATGACTC CAGAGCAAAT GGCTACAAAT GTGAACTGTT CCAGCCCTGA 60

GCGACACACA AGAAGTTATG ATTACATGGA AGGAGGGGAT ATAAGAGTGA GAAGACTCTC 120

TGTCGAACAC AGTGGTACCT GAGGATCGAT AAAAGAGGCA AAGTAAAAGG GACCCAAGAG 180

ATGAAGAATA ATTACAATAT CATGGAAATC AGGACAGTGG CAGTTGGAAT TGTGGCAATC 240

AAAGGGGTGG AAAGTGAATT CTATCTTGCA ATGAACAAGG AAGGAAAACT CTATGCAAAG 300

AAAGAATGCA ATGAAGATTG TAACTTCAAA GAACTAATTC TGGAAAACCA TTACAACACA 360

TATGCATCAG CTAAATGGAC ACACAACGGA GGGGAAATGT TTGTTGCCTT AAATCAAAAG 420

GGGATTCCTG TAAGAGGAAA AAAAACGAAG AAAGAACAAA AAACAGCCCA CTTTCTTCCT 480

ATGGCAATAA CTTAATAGGA TC 502

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 497 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..497)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTATTAAGTT ATTGCCATAG GAAGAAAGTG GGCTGTTTTT TGTTCTTTCT TCGTTTTTTT 60

TCCTCTTACA GGAATCCCCT TTTGATTTAA GGCAACAAAC ATTTCCCCTC CGTTGTGTGT 120

CCATTTAGCT GATGCATATG TGTTGTAATG GTTTTCCAGA ATTAGTTCTT TGAAGTTACA 180

ATCTTCATTG CATTCTTTCT TTGCATAGAG TTTTCCTTCC TTGTTCATTG CAAGATAGAA 240

TTCACTTTCC ACCCCTTTGA TTGCCACAAT TCCAACTGCC ACTGTCCTGA TTTCCATGAT 300

ATTGTAATTA TTCTTCATCT CTTGGGTCCC TTTTACTTTG CCTCTTTTAT CGATCCTCAG 360

GTACCACTGT GTTCGACAGA AGAGTCTTCT CACTCTTATA TCCCCTCCTT CCATGTAATC 420

ATAACTTCTT GTGTGTCGCT CAGGGCTGGA ACAGTTCACA TTTGTAGCCA TTTGCTCTGG 480

AGTCATGTCA TTGCACA 497

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 164 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Cys Asn Asp Met Thr Pro Glu Gln Met Ala Thr Asn Val Asn Cys
1 5 10 15

Ser Ser Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu Gly Gly
20 25 30

Asp Ile Arg Val Arg Arg Leu Phe Cys Arg Thr Gln Trp Tyr Leu Arg
35 40 45

Ile Asp Lys Arg Gly Lys Val Lys Gly Thr Gln Glu Met Lys Asn Asn
50 55 60

Tyr Asn Ile Met Glu Ile Arg Thr Val Ala Val Gly Ile Val Ala Ile
65 70 75 80

Lys Gly Val Glu Ser Glu Phe Tyr Leu Ala Met Asn Lys Glu Gly Lys
85 90 95

Leu Tyr Ala Lys Lys Glu Cys Asn Glu Asp Cys Asn Phe Lys Glu Leu
100 105 110

Ile Leu Glu Asn His Tyr Asn Thr Tyr Ala Ser Ala Lys Trp Thr His
115 120 125

Asn Gly Gly Glu Met Phe Val Ala Leu Asn Gln Lys Gly Ile Pro Val
130 135 140

Arg Gly Lys Lys Thr Lys Lys Glu Gln Lys Thr Ala His Phe Leu Pro
145 150 155 160

Met Ala Ile Thr

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 503 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TATGTGCAAT GACATGACTC CAGAGCAAAT GGCTACAAAT GTGAACTGTT CCAGCCCTGA 60

GCGACACACA AGAAGTTATG ATTACATGGA AGGAGGGGAT ATAAGAGTGA GAAGACTCTT 120

CTGTCGAACA CAGTGGTACC TGCGTATCGA CAAACGCGGC AAAGTCAAGG GCACCCAAGA 180

GATGAAAAAC AACTACAATA TTATGGAAAT CCGTACTGTT GCTGTTGGTA TCGTTGCAAT 240

CAAAGGTGTT GAATCTGAAT TCTATCTTGC AATGAACAAG GAAGGAAAAC TCTATGCAAA 300

GAAAGAATGC AATGAAGATT GTAACTTCAA AGAACTAATT CTGGAAAACG GTTACAACAC 360

ATATGCATCA GCTAAATGGA CACACAACGG AGGGGAAATG TTTGTTGCCT TAAATCAAAA 420

GGGGATTCCT GTAAGAGGAA AAAAAACGAA GAAAGAACAA AAAACAGCCC ACTTTCTTCC 480

TATGGCAATA ACTTAATAGG ATC 503

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 497 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..497)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTATTAAGTT ATTGCCATAG GAAGAAAGTG GGCTGTTTTT TGTTCTTTCT TCGTTTTTTT 60

TCCTCTTACA GGAATCCCCT TTTGATTTAA GGCAACAAAC ATTTCCCCTC CGTTGTGTGT 120

CCATTTAGCT GATGCATATG TGTTGTAACC GTTTTCCAGA ATTAGTTCTT TGAAGTTACA 180

ATCTTCATTG CATTCTTTCT TTGCATAGAG TTTTCCTTCC TTGTTCATTG CAAGATAGAA 240

TTCAGATTCA ACACCTTTGA TTGCAACGAT ACCAACAGCA ACAGTACGGA TTTCCATAAT 300

ATTGTAGTTG TTTTTCATCT CTTGGGTGCC CTTGACTTTG CCGCGTTTGT CGATACGCAG 360

GTACCACTGT GTTCGACAGA AGAGTCTTCT CACTCTTATA TCCCCTCCTT CCATGTAATC 420

ATAACTTCTT GTGTGTCGCT CAGGGCTGGA ACAGTTCACA TTTGTAGCCA TTTGCTCTGG 480

AGTCATGTCA TTGCACA 497

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 164 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Cys Asn Asp Met Thr Pro Glu Gln Met Ala Thr Asn Val Asn Cys
1 5 10 15

Ser Ser Pro Glu Arg His Thr Arg Ser Tyr Asp Tyr Met Glu Gly Gly
20 25 30

Asp Ile Arg Val Arg Arg Leu Phe Cys Arg Thr Gln Trp Tyr Leu Arg
35 40 45

Ile Asp Lys Arg Gly Lys Val Lys Gly Thr Gln Glu Met Lys Asn Asn
50 55 60

Tyr Asn Ile Met Glu Ile Arg Thr Val Ala Val Gly Ile Val Ala Ile
65 70 75 80

Lys Gly Val Glu Ser Glu Phe Tyr Leu Ala Met Asn Lys Glu Gly Lys
85 90 95

Leu Tyr Ala Lys Lys Glu Cys Asn Glu Asp Cys Asn Phe Lys Glu Leu
100 105 110

Ile Leu Glu Asn Gly Tyr Asn Thr Tyr Ala Ser Ala Lys Trp Thr His
115 120 125

Asn Gly Gly Glu Met Phe Val Ala Leu Asn Gln Lys Gly Ile Pro Val
130 135 140

Arg Gly Lys Lys Thr Lys Lys Glu Gln Lys Thr Ala His Phe Leu Pro
145 150 155 160

Met Ala Ile Thr

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGATTTGATT CTAGAAGGAG GAATAACATA TGGTTAACGC GTTGGAATTC GGTAC 55

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGAATTCCAA CGCGTTAACC ATATGTTATT CCTCCTTCTA GAATCAAAT 49

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAAGAAAACC ATTACAACAC 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACAACGCGTG CAATGACATG ACTCCA 26

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACACATATGT GCAATGACAT GACTCCA 27

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ACAGGATCCT ATTAAGTTAT TGCCATAGGA A 31

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGAAAACGGT TACAACACAT ATGCA 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTGTTGTAAC CGTTTTCCAG AATTAG 26

What is claimed is:

1. An analog of a naturally occurring protein in the FGF family wherein at least one amino acid in the loop-forming sequence of -Asn-His-Tyr-Asn-Thr- in said naturally occurring protein is replaced by a residue of a different amino acid having a higher loop-forming potential.

2. The analog of claim 1 wherein said naturally occurring protein is KGF.

3. The analog of claim 2 wherein said replaced amino acid is amino acid 116.

4. The analog of claim 3 wherein said amino acid having a higher loop-forming potential is selected from the group consisting of glycine, proline, tyrosine, aspartic acid, asparagine, serine, glutamic acid, threonine, lysine, glutamine, arginine, phenylalanine, and tryptophan.

5. The analog of claim 4 wherein said amino acid having a higher loop-forming potential is glycine.

6. The analog of claim 5 having the amino acid sequence set forth in Fig. 10.

7. The analog of claim 6 wherein at least one terminal amino acid residue is deleted while said analog substantially retains said enhanced biological activity.

8. The analog of claim 7 wherein at least one cysteine residue of said naturally occurring KGF is replaced by a residue of a neutral amino acid.

9. A DNA sequence encoding for procaryotic or eucaryotic expression of an analog of claim 1.

10. The DNA sequence of claim 10 wherein said analog is the analog of claim 6.

11. A pharmaceutical composition comprising a therapeutically effective amount of an analog according to claim 1 and one or more pharmaceutically acceptable adjuvants.

12. The pharmaceutical composition of claim 11 wherein said analog is the analog of claim 6.

13. A method for treating a wound comprising administering to said wound a therapeutically effective amount of an analog according to claim 1.

14. The method of claim 13 wherein said analog is the analog of claim 6.

15. The analog of claim 5 having the amino acid sequence set forth in Fig. 1.

16. The analog of claim 5 having the amino acid sequence set forth in Fig. 2.

```
        10                  30                  50
TCTAGAAAAAACCAAGGAGGTAATAAATAATGTTCAACCTGCCGCTGGGTAACTACAAAAAA
                             MetPheAsnLeuProLeuGlyAsnTyrLysLys
                           -1  1                              10

        70                  90                 110
CCTAAGCTTCTGTACTGCTCTAACGGCGGTTACTTCCTGCGCATTCTCCCGGATGGCACT
ProLysLeuLeuTyrCysSerAsnGlyGlyTyrPheLeuArgIleLeuProAspGlyThr
                          20                              30

       130                 150                 170
GTAGACGGTACCAAAGATCGTTCCGACCAGCACATTCAGCTCCAGCTCGCTGCAGAATCT
ValAspGlyThrLysAspArgSerAspGlnHisIleGlnLeuGlnLeuAlaAlaGluSer
                          40                              50

       190                 210                 230
ATCGGTGAAGTTTACATCAAATCCACCGAAACTGGTCAGTTCCTGGCTATGGATACTGAT
IleGlyGluValTyrIleLysSerThrGluThrGlyGlnPheLeuAlaMetAspThrAsp
                          60                              70

       250                 270                 290
GGTCTCCTCTACGGTTCTCAGACTCCGAACGAAGAGTGCCTGTTCCTCGAGCGTCTGGAA
GlyLeuLeuTyrGlySerGlnThrProAsnGluGluCysLeuPheLeuGluArgLeuGlu
                          80                              90

       310                 330                 350
GAAAACGGTTACAACACCTACATCTCCAAAAAACACGCTGAAAAACACTGGTTCGTTGGT
GluAsnGlyTyrAsnThrTyrIleSerLysLysHisAlaGluLysHisTrpPheValGly
                         100                             110

       370                 390                 410
CTGAAAAAAAAACGGTCGTTCTAAACTGGGTCCGCGCACTCACTTCGGTCAGAAAGCTATC
LeuLysLysAsnGlyArgSerLysLeuGlyProArgThrHisPheGlyGlnLysAlaIle
                         120                             130

       430                 450
CTGTTCCTCCCTCTGCCGGTTTCTTCCGATTAATAGGATCC
LeuPheLeuProLeuProValSerSerAspEndEnd
                         140
```

Figure 1

```
-15                 -10                 -5                  -1
Met Ala Glu Gly Glu Ile Thr Thr Phe Thr Ala Leu Thr Glu Lys

 1                   5                  10                  15
Phe Asn Leu Pro Pro Gly Asn Tyr Lys Lys Pro Lys Leu Leu Tyr

                    20                  25                  30
Ala Ser Asn Gly Gly His Phe Leu Arg Ile Leu Pro Asp Gly Thr

                    35                  40                  45
Val Asp Gly Thr Arg Asp Arg Ser Asp Gln His Ile Gln Leu Gln

                    50                  55                  60
Leu Ser Ala Glu Ser Val Gly Glu Val Tyr Ile Lys Ser Thr Glu

                    65                  70                  75
Thr Gly Gln Tyr Leu Ala Met Asp Thr Asp Gly Leu Leu Tyr Gly

                    80                  85                  90
Ser Gln Thr Pro Asn Glu Glu Cys Leu Phe Leu Glu Arg Leu Glu

                    95                 100                 105
Glu Asn Gly Tyr Asn Thr Tyr Ile Ser Lys Lys His Ala Glu Lys

                   110                 115                 120
Asn Trp Phe Val Gly Leu Lys Lys Asn Gly Ser Cys Lys Arg Gly

                   125                 130                 135
Pro Arg Thr His Tyr Gly Gln Lys Ala Ile Leu Phe Leu Pro Leu

                   140
Pro Val Ser Ser Asp
```

Figure 2

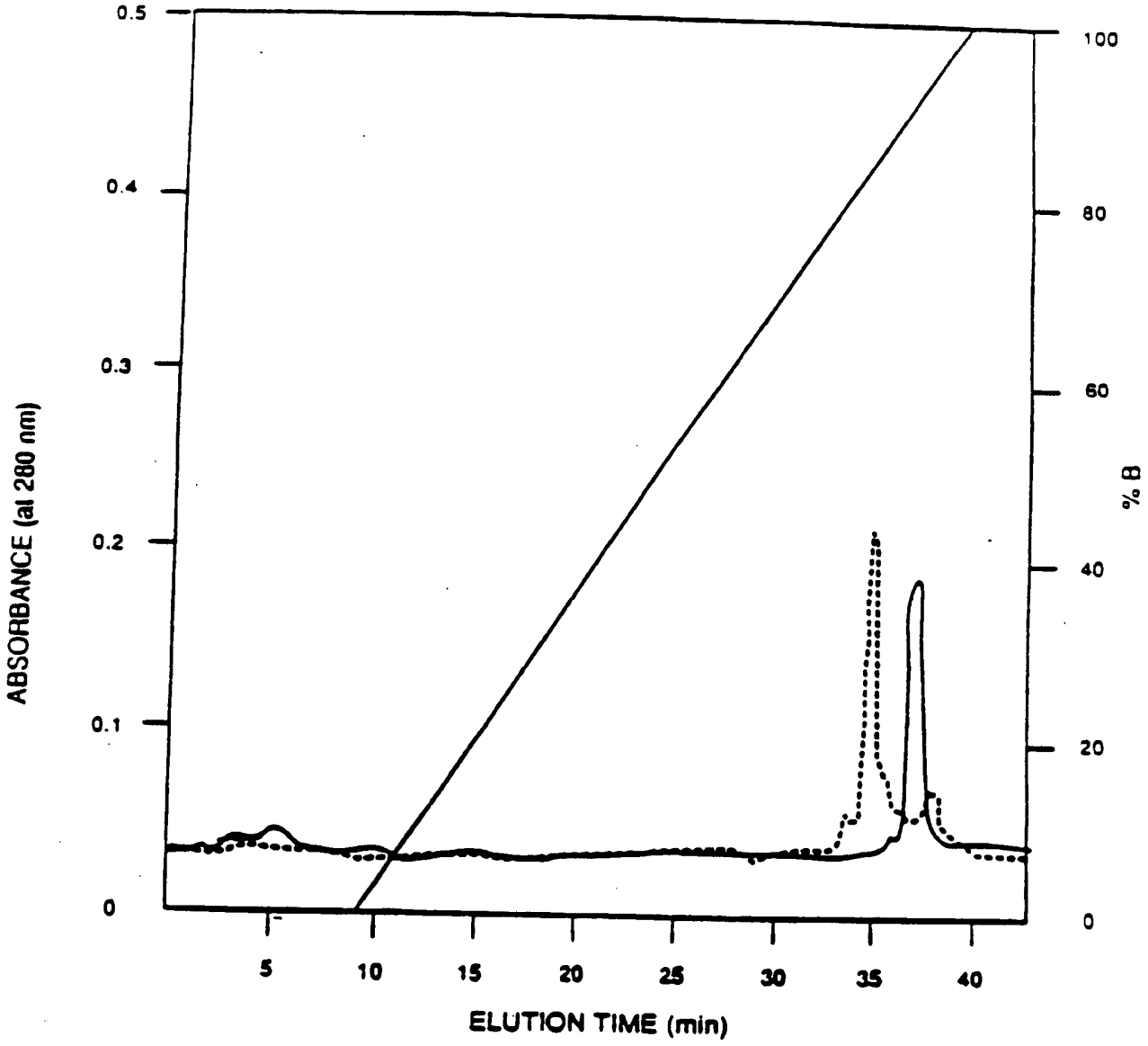

0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (at 280 nm)
100
80
60
40
20
0
% B
5
10
15
20
25
30
35
40
ELUTION TIME (min)
[Ala 47, Gly 93] aFGF
[Ala 47] aFGF

FIGURE 3

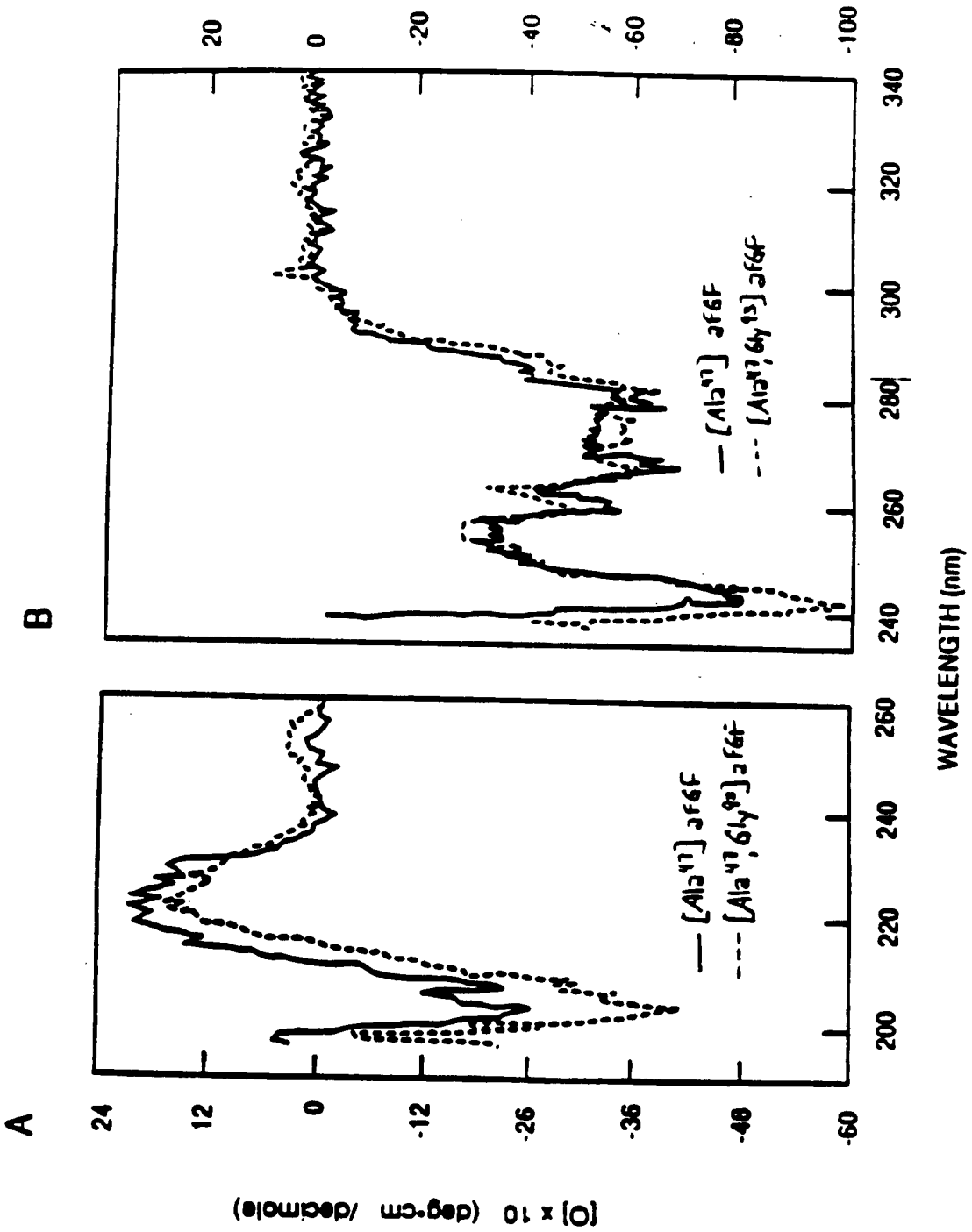

A
B
[Θ] x 10 (deg·cm /decimole)
WAVELENGTH (nm)
— [Ala17] 3f6F
--- [Ala17,Gly19] 3f6F
— [Ala17] 3f6F
--- [Ala17,Gly13] 3f6F

FIGURE 4

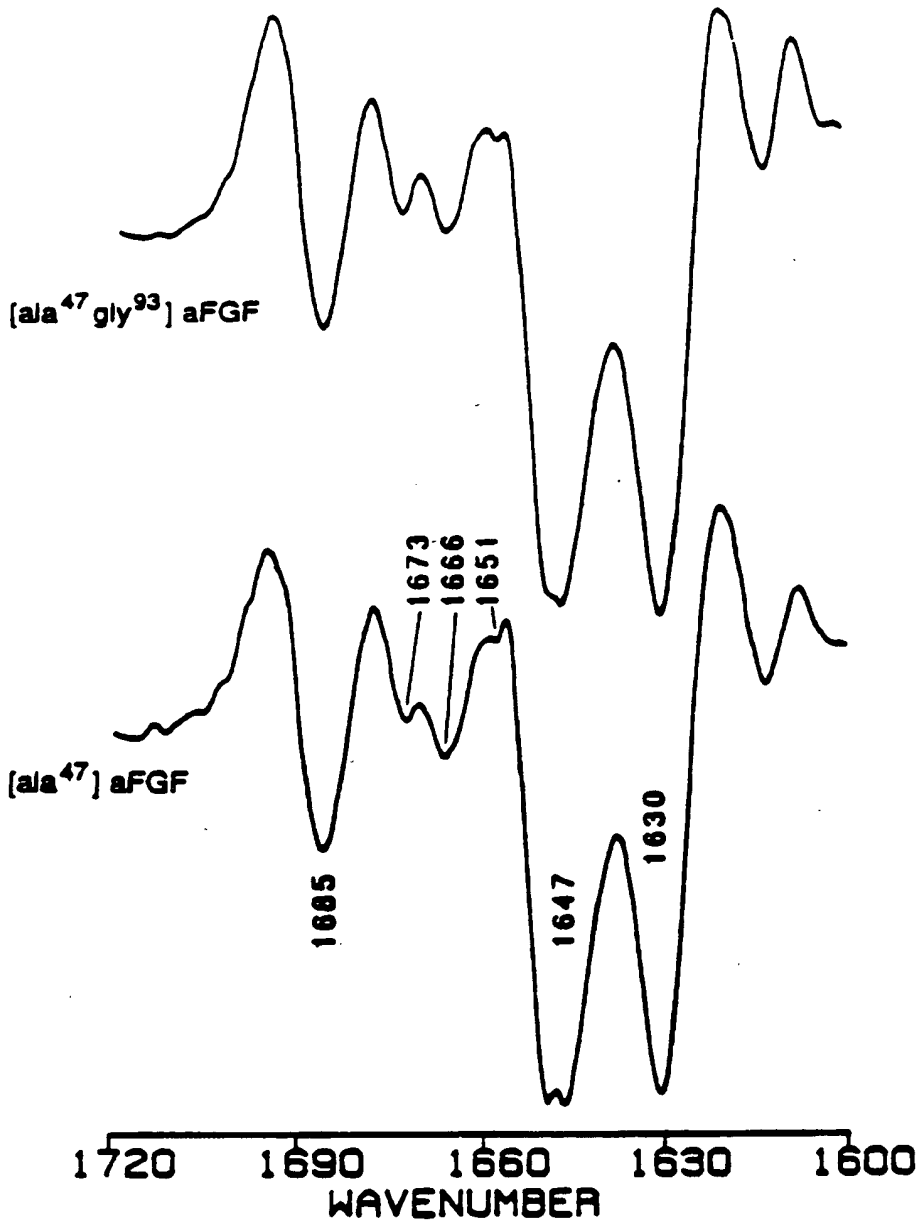

[ala47 gly93] aFGF
[ala47] aFGF
1673
1666
1651
1685
1647
1630
1720
1690
1660
1630
1600
WAVENUMBER

FIGURE 5

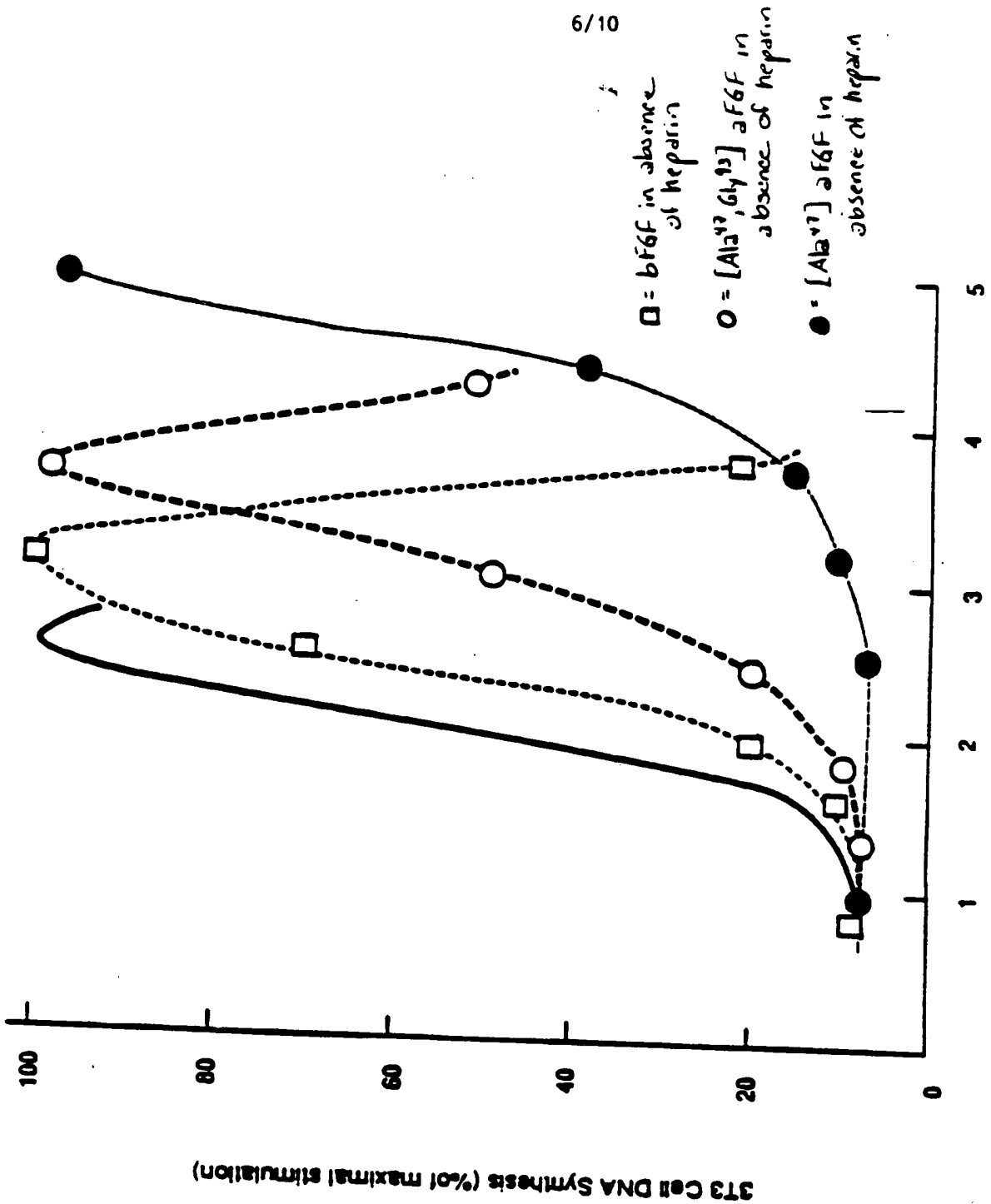

□ = bFGF in absence of heparin
○ = [Ala^31, Gly^33] aFGF in absence of heparin
● = [Ala^31] aFGF in absence of heparin
3T3 Cell DNA Synthesis (%of maximal stimulation)
100
80
60
40
20
0
1
2
3
4
5
log FGF (pg/ml)

FIGURE 6

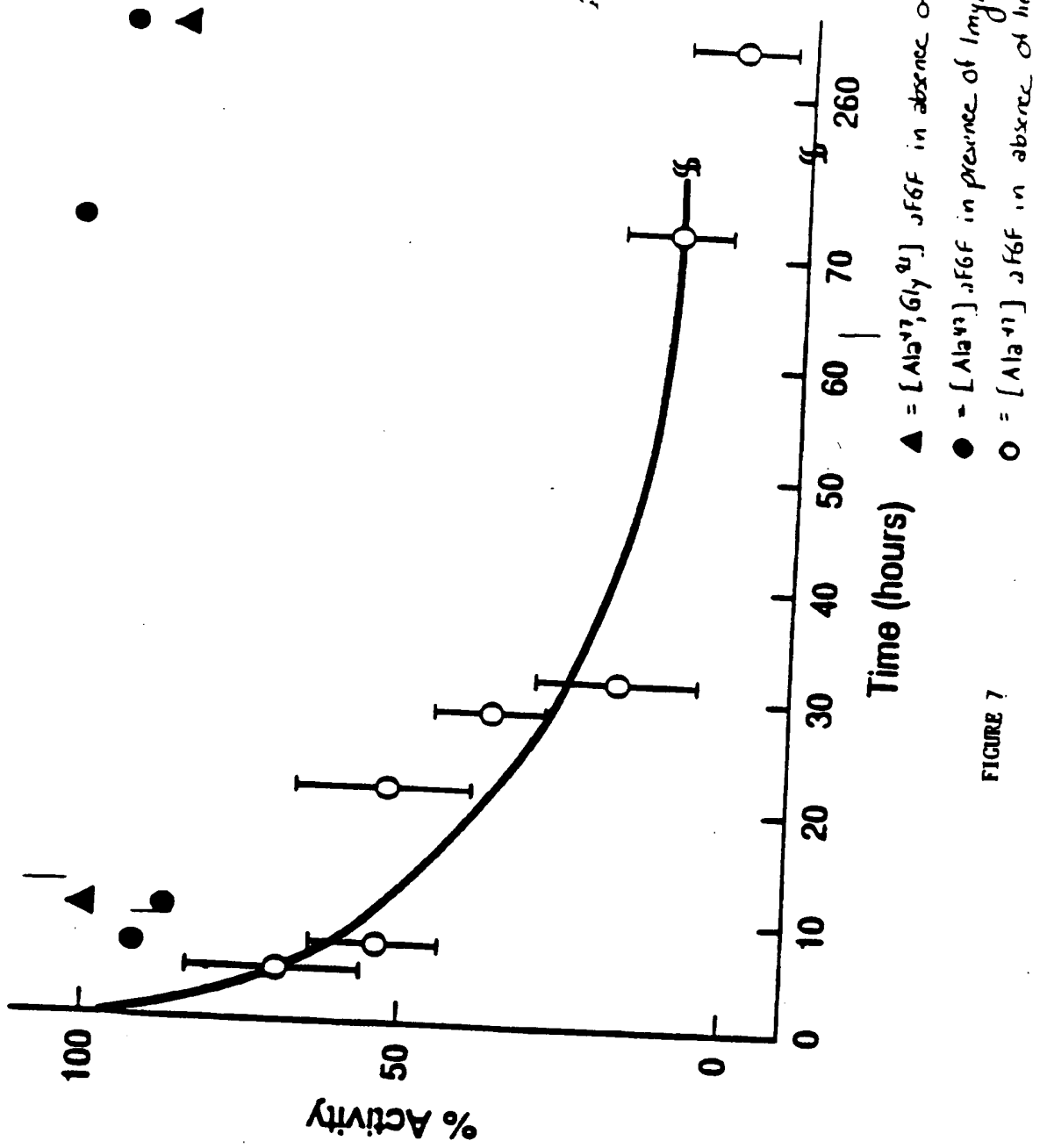

% Activity
100
50
0
0
10
20
30
40
50
60
70
260
Time (hours)
▲ = [Ala⁴¹, Gly⁹⁶] aFGF in absence of hepar
● = [Ala⁴¹] aFGF in presence of lmyfal hep
○ = [Ala⁴¹] aFGF in absence of heparin

FIGURE 7

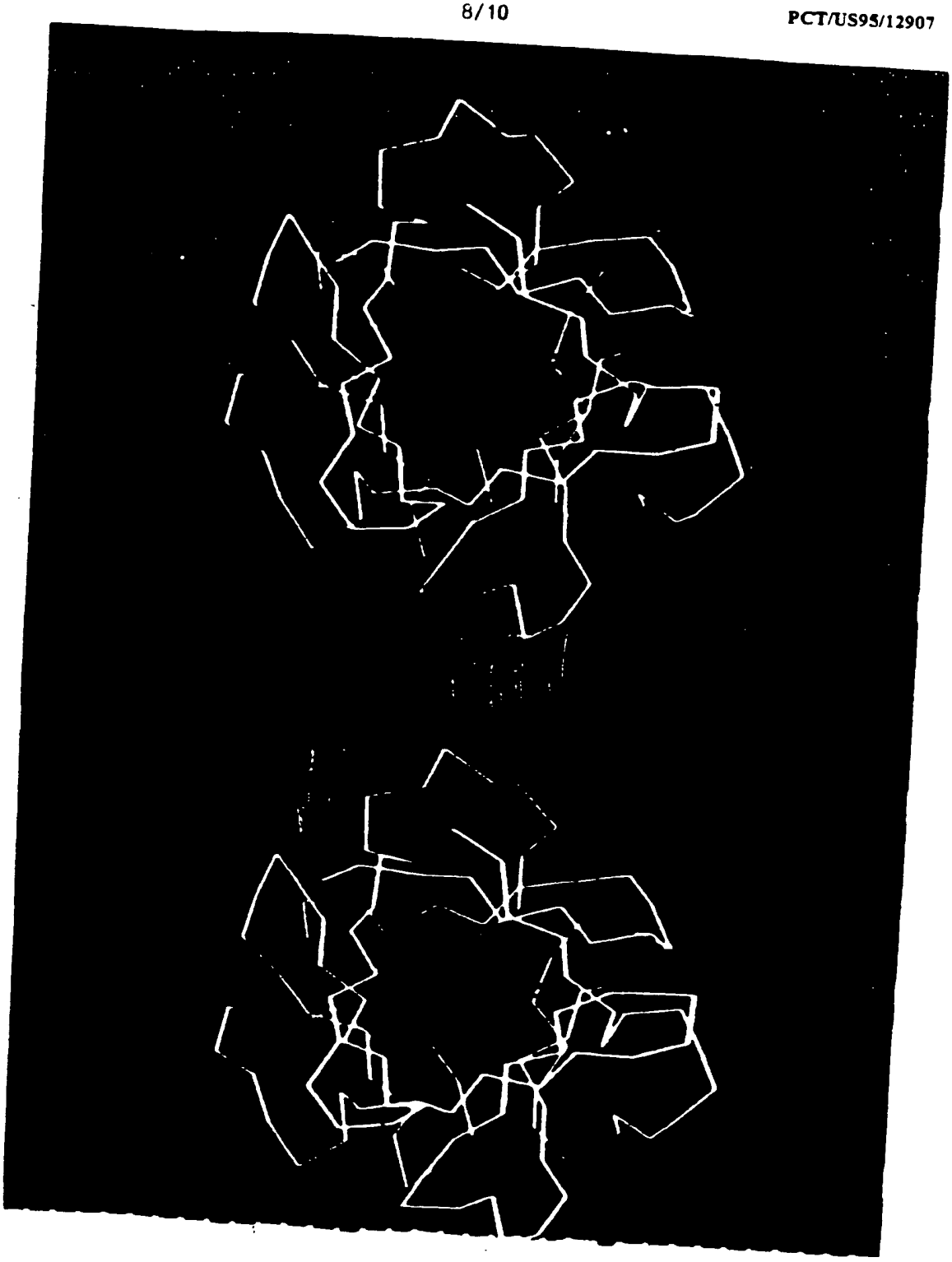


FIGURE 8

Figure 9

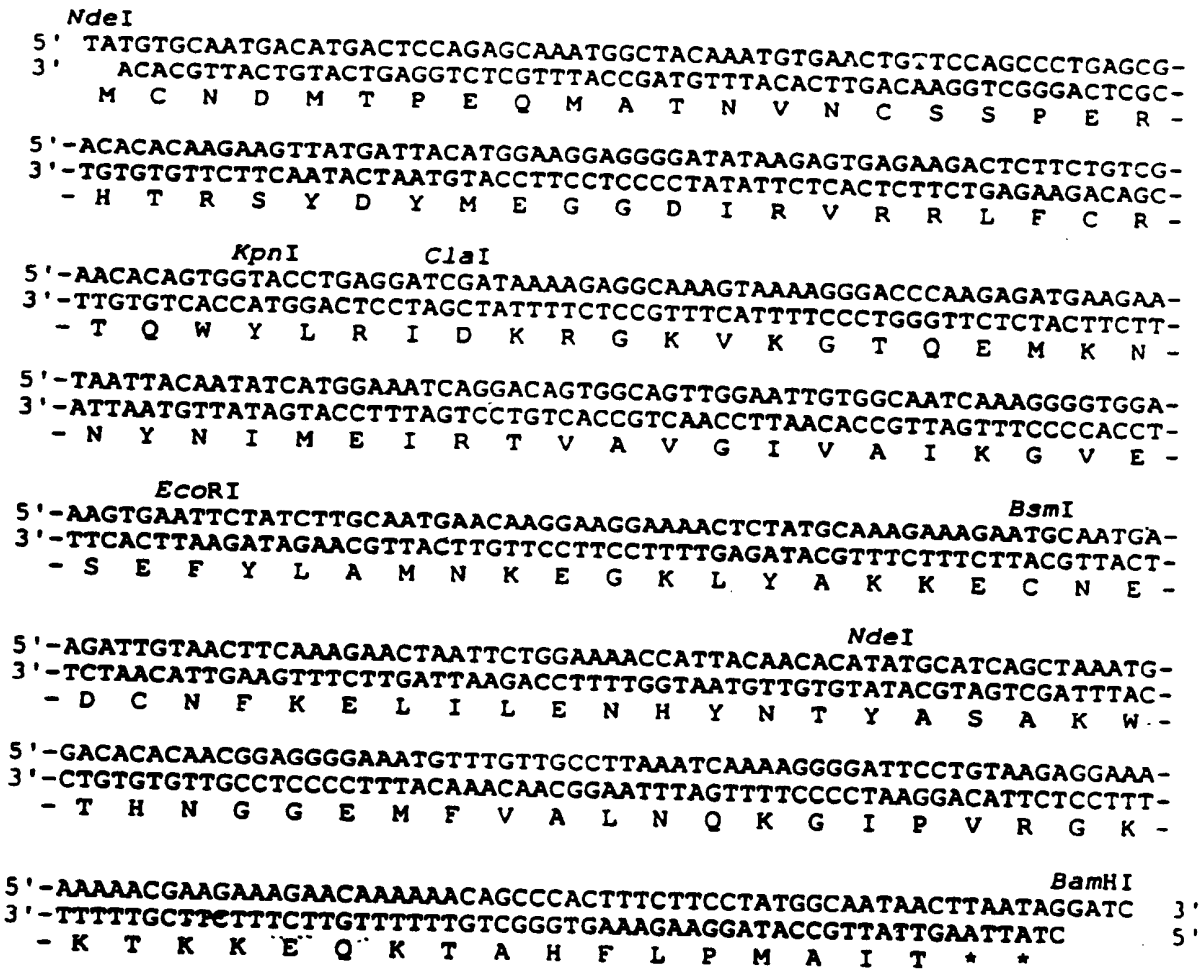
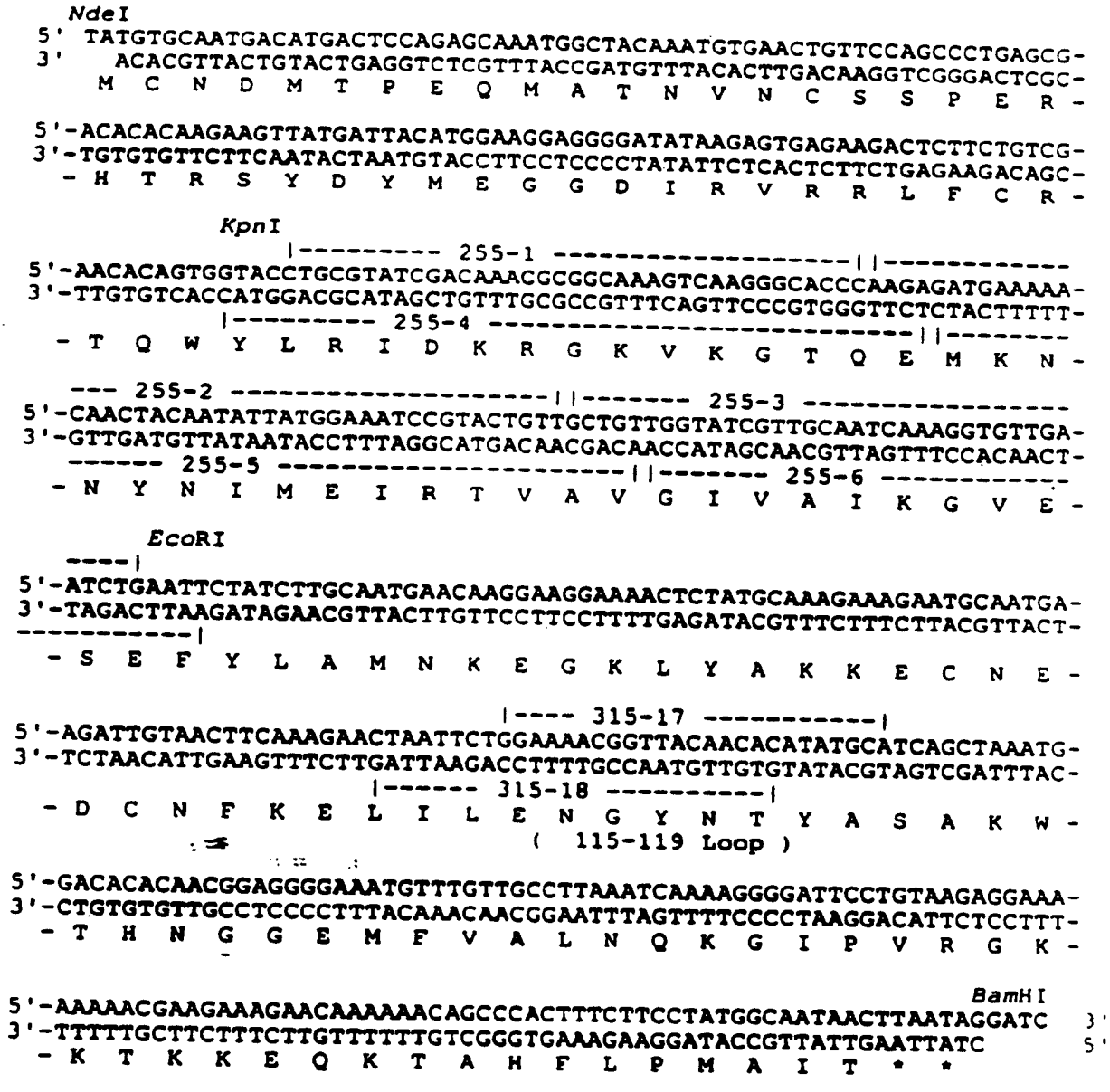

NdeI
5' TATGTGCAATGACATGACTCCAGAGCAAATGGCTACAAATGTGAACTGTTCCAGCCCTGAGCG-
3' ACACGTTACTGTACTGAGGTCTCGTTTACCGATGTTTACACTTGACAAGGTCGGGACTCGC-
M C N D M T P E Q M A T N V N C S S P E R -
5'-ACACACAAGAAGTTATGATTACATGGAAGGAGGGGATATAAGAGTGAGAAGACTCTTCTGTCG-
3'-TGTGTGTTCTTCAATACTAATGTACCTTCCTCCCCTATATTCTCACTCTTCTGAGAAGACAGC-
- H T R S Y D Y M E G G D I R V R R L F C R -
KpnI
ClaI
5'-AACACAGTGGTACCTGAGGATCGATAAAAGAGGCAAAGTAAAAGGGACCCAAGAGATGAAGAA-
3'-TTGTGTCACCATGGACTCCTAGCTATTTTCTCCGTTTCATTTTCCCTGGGTTCTCTACTTCTT-
- T Q W Y L R I D K R G K V K G T Q E M K N -
5'-TAATTACAATATCATGGAAATCAGGACAGTGGCAGTTGGAATTGTGGCAATCAAAGGGGTGGA-
3'-ATTAATGTTATAGTACCTTTAGTCCTGTCACCGTCAACCTTAACACCGTTAGTTTCCCCACCT-
- N Y N I M E I R T V A V G I V A I K G V E -
EcoRI
BsmI
5'-AAGTGAATTCTATCTTGCAATGAACAAGGAAGGAAAACTCTATGCAAAGAAAGAATGCAATGA-
3'-TTCACTTAAGATAGAACGTTACTTGTTCCTTCCTTTTGAGATACGTTTCTTTCTTACGTTACT-
- S E F Y L A M N K E G K L Y A K K E C N E -
NdeI
5'-AGATTGTAACTTCAAAGAACTAATTCTGGAAAACCATTACAACACATATGCATCAGCTAAATG-
3'-TCTAACATTGAAGTTTCTTGATTAAGACCTTTTGGTAATGTTGTGTATACGTAGTCGATTTAC-
- D C N F K E L I L E N H Y N T Y A S A K W -
5'-GACACACAACGGAGGGGAAATGTTTGTTGCCTTAAATCAAAAGGGGATTCCTGTAAGAGGAAA-
3'-CTGTGTGTTGCCTCCCCTTTACAAACAACGGAATTTAGTTTTCCCCTAAGGACATTCTCCTTT-
- T H N G G E M F V A L N Q K G I P V R G K -
BamHI
5'-AAAAACGAAGAAAGAACAAAAAACAGCCCACTTTCTTCCTATGGCAATAACTTAATAGGATC 3'
3'-TTTTTGCTTCTTTCTTGTTTTTTGTCGGGTGAAAGAAGGATACCGTTATTGAATTATC 5'
- K T K K E Q K T A H F L P M A I T * *

Figure 10


NdeI
5' TATGTGCAATGACATGACTCCAGAGCAAATGGCTACAAATGTGAACTGTTCCAGCCCTGAGCG-
3' ACACGTTACTGTACTGAGGTCTCGTTTACCGATGTTTACACTTGACAAGGTCGGGACTCGC-
M C N D M T P E Q M A T N V N C S S P E R -
5'-ACACACAAGAAGTTATGATTACATGGAAGGAGGGGATATAAGAGTGAGAAGACTCTTCTGTCG-
3'-TGTGTGTTCTTCAATACTAATGTACCTTCCTCCCCTATATTCTCACTCTTCTGAGAAGACAGC-
- H T R S Y D Y M E G G D I R V R R L F C R -
KpnI
255-1
5'-AACACAGTGGTACCTGCGTATCGACAAACGCGGCAAAGTCAAGGGCACCCAAGAGATGAAAAA-
3'-TTGTGTCACCATGGACGCATAGCTGTTTGCGCCGTTTCAGTTCCCGTGGGTTCTCTACTTTTT-
255-4
- T Q W Y L R I D K R G K V K G T Q E M K N -
255-2
255-3
5'-CAACTACAATATTATGGAAATCCGTACTGTTGCTGTTGGTATCGTTGCAATCAAAGGTGTTGA-
3'-GTTGATGTTATAATACCTTTAGGCATGACAACGACAACCATAGCAACGTTAGTTTCCACAACT-
255-5
255-6
- N Y N I M E I R T V A V G I V A I K G V E -
EcoRI
5'-ATCTGAATTCTATCTTGCAATGAACAAGGAAGGAAAACTCTATGCAAAGAAAGAATGCAATGA-
3'-TAGACTTAAGATAGAACGTTACTTGTTCCTTCCTTTTGAGATACGTTTCTTTCTTACGTTACT-
- S E F Y L A M N K E G K L Y A K K E C N E -
315-17
5'-AGATTGTAACTTCAAAGAACTAATTCTGGAAAACGGTTACAACACATATGCATCAGCTAAATG-
3'-TCTAACATTGAAGTTTCTTGATTAAGACCTTTTGCCAATGTTGTGTATACGTAGTCGATTTAC-
315-18
- D C N F K E L I L E N G Y N T Y A S A K W -
(115-119 Loop)
5'-GACACACAACGGAGGGGAAATGTTTGTTGCCTTAAATCAAAAGGGGATTCCTGTAAGAGGAAA-
3'-CTGTGTGTTGCCTCCCCTTTACAAACAACGGAATTTAGTTTTCCCCTAAGGACATTCTCCTTT-
- T H N G G E M F V A L N Q K G I P V R G K -
BamHI
5'-AAAAACGAAGAAAGAACAAAAAACAGCCCACTTTCTTCCTATGGCAATAACTTAATAGGATC 3'
3'-TTTTTGCTTCTTTCTTGTTTTTTGTCGGGTGAAAGAAGGATACCGTTATTGAATTATC 5'
- K T K K E Q K T A H F L P M A I T * *

INTERNATIONAL SEARCH REPORT

Interr ...al Application No
PCT/US 95/12907

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/50 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 11360 (AMGEN INC.) 9 July 1992 see page 4, line 27 - page 5, line 3 see page 6, line 7 - page 8, line 2 see page 10, line 31 - page 14, line 7; figures 1,2 ---	1,9,15, 16
X	PROTEIN ENGINEERING, vol. 6, no. 5, July 1993 ENGLAND GB, pages 541-546, TSUTOMO ARAKAWA ET AL. 'Production and characterization of an analog of acidic fibroblast growth factor with enhanced stability and biological activity' see abstract see page 545, right column, paragraph 3 - page 546, left column, paragraph 1 -----	1,9,15, 16

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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'&' document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
11 January 1996	05. 03. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/12907

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9211360	09-07-92	AU-B-	663067	28-09-95
		AU-B-	9152491	22-07-92
		EP-A-	0575339	29-12-93
		JP-T-	6503962	12-05-94
		NZ-A-	241021	23-12-93